Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains

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This study examined the relationship between blastomere fragmentation in cultured human embryos obtained by in-vitro fertilization and the effect of fragmentation on the distribution of the following eight regulatory proteins found to be: (i) localized in the mature oocyte in subplasmalemmal, polarized domains; and (ii) unequally inherited by the blastomeres during cleavage: leptin, signal transducer and activator of transcription 3 (STAT3), Bax, Bcl-x, transforming growth factor β2 (TGFβ2), vascular endothelial growth factor (VEGF), c-kit and epidermal growth factor R (EGF-R). Four basic patterns of fragmentation were observed. The severity of the impact of each type of fragmentation on the affected blastomere(s) and the developmental competence of the embryo appeared to be a function of the unique temporal and spatial features associated with the particular fragmentation pattern(s) involved in each instance. The findings demonstrate that certain patterns of fragmentation can result in the partial or near total loss of the eight regulatory proteins from specific blastomeres and that the developmental potential of the affected embryo can be particularly compromised if it occurs during the 1- or 2-cell stages. In contrast, fragmentation from portions of a fertilized egg or a blastomere(s) in a 2-cell embryo that do not contain the protein domains, or the complete loss by fragmentation of a regulatory protein domain-containing blastomere after the 4-cell stage does not necessarily preclude continued development to the blastocyst, although the normality and developmental potential of the embryo may be compromised. The possible association between fragmentation and apoptosis was examined by annexin V staining of plasma membrane phosphatidylserine and TUNEL analysis of blastomere DNA. No direct correlation between fragmentation and apoptosis was found following the analyses of fragmented embryos with these two markers. However, while we suggest that changes in cell physiology unrelated to apoptosis are the more likely causes of fragmentation, we cannot exclude the possibility that fragmentation itself may be an initiator of apoptosis if critical ratios or levels of developmentally important proteins are altered by partial or complete elimination of their polarized domains. The findings are discussed with respect to the possible developmental significance of regulatory protein polarization in human oocytes and preimplantation stage embryos.

Key words: apoptosis/developmental competence/embryo fragmentation/polarized protein domains/preimplantation development

Introduction

The complete or partial fragmentation of one or more blastomeres resulting in conversion to a pleiomorphic population of cytoplasts is a common occurrence during the early cleavage stages of human embryonic development *in vitro*. A very similar phenomenon has been reported to occur with human embryos fertilized *in vivo* (Buster *et al.*, 1985). Both apoptotic and necrotic processes have been suggested as causes of blastomere fragmentation in human embryos (Juriscova *et al.*, 1996), but a definitive aetiology has yet to be determined. In this regard, it remains unclear whether all forms and degrees of fragmentation are indications that the competence of an affected cell(s) or the entire embryo has been necessarily compromised. Outcome data from some clinical in-vitro fertilization (IVF) studies suggests that embryo developmental potential declines significantly as the number of cytoplasmic fragments increases (Giorgetti *et al.*, 1995), while others have shown no significant correlation (Hoover *et al.*, 1995). These empirically based findings have led to the incorporation of estimates of fragment number into schemes designed to assess developmental competence prior to embryo transfer or cryopreservation (Giorgetti *et al.*, 1995; for review see Van Blerkom, 1997).

We have shown previously that the regulatory proteins leptin and STAT3 occur in polarized domains in both mouse and human oocytes and that, after fertilization, these domains become differentially distributed between blastomeres in the cleavage stage embryo and between the inner cell mass and trophoblast of the blastocyst (Antczak and Van Blerkom, 1997). The position of these domains in the immature oocyte appears to delineate the future animal pole, which is defined as the region of first polar body abstriction, as well as the future embryonic/abembryonic axis, which is associated with the region of the penetrated egg from which the second polar body emerges (Gardner, 1997). In the case of leptin and STAT3, the pattern of inheritance of the polarized domains in daughter blastomeres seems to be determined by how successive equatorial or meridional planes of cell division (Edwards

and Beard, 1997) are oriented with respect to the domains. We have suggested that the subplasmalemmal localization of the leptin and STAT3 domains within individual blastomeres may have clinical relevance in understanding the variable developmental potential of fragmented human embryos. For a particular blastomere(s), fragmentation from these domains may reduce or deplete the complement of these or other regulatory proteins such that while capable of cytokinesis, developmental viability may be impaired or eliminated.

The purpose of the present study was to determine the qualitative effects of fragmentation on cortically positioned domains of regulatory proteins during the first 72 h of human embryo culture. In addition to a re-examination of leptin and STAT3, we report that members of the following classes of proteins are also found in polarized domains in human oocytes and embryos and that they too, as a result of their localization, are affected by the fragmentation process: (i) growth factor receptors [c-erbB (epidermal growth factor receptor (EGF-R)] and c-kit (stem cell factor receptor); (ii) apoptosis proteins (Bcl-x and Bax); and (iii) growth factors [transforming growth factor β2 (TGFβ2) and vascular endothelial growth factor (VEGF)]. During these analyses, four distinct patterns of fragmentation were observed, the consequences of each being directly affected by the particular spatial and temporal manifestations impacting a particular egg or embryo. In the majority of cleavage stage embryos that contained blastomeres with cytoplasmic fragments, immunofluorescent analysis by scanning laser confocal microscopy demonstrated that these structures frequently formed from the portion of the plasma membrane associated with the regulatory protein domains, and that as a result of the incorporation of portions of the affected domain(s) into the cytoplasmic extrusions, the apparent complement of these proteins was either reduced or undetectable in the affected blastomere(s). These findings suggest that the differential developmental potential exhibited by fragmented human embryos may be associated with a blastomere-specific depletion of critical regulatory proteins that is determined both by the specific pattern of fragmentation and the specific portion of the plasma membrane and subjacent cytoplasm involved.

The extent to which apoptotic processes may be associated with different patterns of fragmentation and disruption of the polarized domains was examined in fragmented embryos by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) to detect 3′-OH DNA strand breaks, and by annexin V staining to detect translocation of phosphatidylserine from the inner to the outer aspects of the plasma membrane. Our findings indicate that fragmentation *per se* does not appear to be part of, nor a direct consequence of, an apoptotic process. However, we cannot exclude the possibility that some forms of fragmentation can lead to apoptosis by virtue of their effects on cortically localized organelles and/or the complements of important polarized protein domains, in particular those involving apoptosis-associated proteins.

Materials and methods

Oocytes and embryos

Fragmented embryos examined in this study were obtained from stimulated cycles (Van Blerkom *et al.*, 1995a) that involved conven-

tional IVF for women between the ages of 23 and 41 years (mean 36 years). Oocytes were exposed to 3000–10 000 motile spermatozoa for 3 h, transferred to fresh medium, and inspected for the presence of pronuclei between 8 and 12 h after insemination as previously described (Van Blerkom *et al.*, 1995a). All oocytes and embryos were cultured in an atmosphere of 4.5% O_2, 5.5% CO_2 and 90% N_2. Fertilized eggs were examined at 8- to 12-h intervals during the following 60–65 h of culture, at the end of which time embryo transfer was performed (72–77 h post insemination). If cytoplasmic fragments were observed, this inspection schedule permitted, within 12 h intervals, a determination of the pattern, relative size, location, approximate number and embryo stage at which fragmentation occurred. Because these characteristics were recorded on videotape at each inspection, changes in fragment number or location could be documented. According to protocol, embryos that fragmented at the 2- (22–26 h) or 4-cell stages (34–38 h) and which failed to undergo additional cleavage divisions during the subsequent 24–26 h of culture were considered to have arrested development and, with patient permission, were used for analysis. Embryos that had fragmented during the first 36 h of culture but which progressed to the 8-cell stage were used (with patient permission) if cryopreservation was not an option. Normal-appearing early cleavage stage embryos were derived from dispermic fertilizations or, if monospermic, were donated by patients who requested that only a predetermined number of embryos be replaced and any remaining embryos not be cryopreserved.

Fixation and analysis by scanning laser confocal immunofluorescence

A small portion of the human oocytes and embryos examined were exposed briefly (20 s) to acidic Tyrode's solution for zona thinning prior to fixation. All oocytes and embryos were fixed in a phosphate-buffered saline (PBS) solution containing 3.7% formaldehyde (pH 7.3) for 1 h at room temperature. After fixation: (i) the residual zona was removed mechanically by repeated passage through a narrow-bore glass micropipette (zona on 'thinned' oocytes or embryos); (ii) intact zonae from oocytes not exposed to Tyrode's solution were removed mechanically by repeated passage through a narrow-bore glass micropipette; or (iii) intact zonae were removed mechanically by dissection with fine tungsten needles (embryos only). After removal of the zona pellucida, specimens were permeabilized in PBS solution containing 0.1% Triton X-100 and 0.1% NP-40 (Sigma Chemical Co., St Louis, MO, USA) for 1 h at room temperature. The protocols for antibody staining and confocal microscopy have been described in detail previously (Antczak and Van Blerkom, 1997). In brief, oocytes and embryos were reacted with affinity-purified, primary antibody solutions directed against leptin [Ob (Y20)], STAT3 (C-20), TGFβ2 (V), EGF-R (1005), c-kit (C-19, 4 μg/ml), Bax (I-19), Bcl-x (S-18), VEGF (C1, 2 μg/ml) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), actin (A2066, 1:100 dilution) and uvomorulin (E-cadherin, U3254, 1:200 dilution) (both from Sigma Immunochemicals, St Louis, MO, USA), prepared in PBS with 2% bovine serum albumin (PBS–BSA) at a concentration of 1 μg/ml, unless otherwise specified. For rabbit polyclonal primary antibodies involved in single antibody analysis, a 1:200 dilution of goat anti-rabbit fluorescein isothiocyanate (FITC) conjugate (Sigma Immunochemicals) prepared in PBS–BSA was used as secondary antibody solution. For rat polyclonal primary antibodies a 1:150 dilution of a rabbit anti-rat FITC conjugate (Sigma Immunochemicals) prepared in PBS–BSA was used as secondary antibody solution. In the case of the VEGF antibody, a 1:100 dilution of a goat anti-mouse FITC conjugate (Sigma Immunochemicals) prepared in PBS with 2% BSA was used as secondary antibody. In instances where oocytes and embryos were stained simultaneously with antibodies directed against

VEGF and c-kit, or VEGF and Bcl-x, primary antibodies were used at the same concentrations as for single-antibody analyses. However, under these conditions while reactivity of the VEGF antibody was detected as described for singly stained specimens, a 1:200 dilution of a goat anti-rabbit biotinylated antibody (Sigma) prepared in PBS–BSA was used as secondary antibody against the rabbit primary antibody, followed (after washing) by incubation of the samples in a 1:100 dilution of streptavidin–Texas red (Molecular Probes, Eugene, OR, USA) for 2 h at 4°C. To serve as controls during dual-stained analyses, samples singly stained with FITC-conjugated secondary antibody (VEGF) were scanned using settings and filter combinations designed to detect both FITC and Texas red (dual-analysis filters and settings); samples singly stained with Texas red (actin, Bax) were scanned using settings and filter combinations designed to detect both Texas red and FITC to determine the amount of overlap present in the emissions from the two fluorochromes during dual-staining analysis. Under the conditions of the analysis, no overlap was detected between FITC- and Texas red-associated fluorescent signals (results not shown). All images obtained by light, epifluorescence or scanning laser confocal microscopy are presented as observed during examination, without any enhancement or attenuation of the fluorescent signals.

To confirm the specificity of the antibodies used during these analyses, each antibody solution was preincubated with the immunizing peptide (leptin, SC-843P; STAT3, SC-482P; TGFβ2, SC-90P; EGF-R, SC-03P; c-kit, SC-168p; Bax, SC-526P; Bcl-x, SC-634P; Santa Cruz Biotechnology) at a concentration 20-fold that used with the primary antibody, for 1–2 h at 20°C, with the exception of the VEGF antibody for which a blocking peptide was unavailable. Following this preincubation period, unfertilized oocytes were processed as described previously for singly stained specimens (Antczak and Van Blerkom, 1997) and examined for associated immunofluorescence. To confirm the spatial specificity of the fluorescent signal(s), representative specimens were placed between coverslips, examined by confocal microscopy, and rescanned with the specimen inverted. To control for non-specific immunofluorescence, human and mouse oocytes and fragmented embryos were reacted with mouse or rabbit IgG antibodies (sc-2025 and sc-2027; Santa Cruz Biotechnology) derived from non-immunized animals at concentrations equivalent to those used for specific primary antibodies, followed by incubation in the appropriate FITC- and/or biotin-conjugated secondary antibody at the same concentrations and times as described above.

Colorimetric antibody analysis

In several instances the protein domains associated with particular oocytes, embryos and fragmented embryos as detected by immunofluorescent analyses were re-examined using a colorimetric antibody detection protocol to confirm the positioning and distribution of those domains. To assist with the repositioning of specimens for direct comparison of results following the second antibody detection protocol, an 18 mm coverslip was applied to the droplets of Slow Fade containing the specimens on a 22×40 mm coverglass prior to the immunofluorescent analysis step. Application of the coverslip resulted in the compression of the specimen(s) between the glass surfaces and created two relatively flat surfaces, which facilitated re-establishment of the original sample orientation during subsequent analyses. For colorimetric analysis, samples were removed from Slow Fade, rehydrated in PBS containing 1% BSA, and then re-exposed to primary antibody solutions as described previously. Samples were then incubated with a 1:80 dilution of goat anti-rabbit horseradish peroxidase (HRP)-conjugate (SC2004; Santa Cruz Biotechnology Inc.) in PBS–BSA for 2 h at 4°C. Following a series of three washes in PBS containing 1% BSA, samples were transferred to a solution containing SIGMA FAST DAB with metal enhancer (D0426; Sigma

Immunochemicals), prepared according to the manufacturer's instructions. When optimal colour development was achieved, samples were transferred to PBS containing 1% BSA and 0.1% sodium azide and transferred through three washes to remove the colorimetric substrate and inactivate peroxidase activity. In each case, experimental and control samples were processed in an identical fashion, for the same periods of time. Results were obtained with a camera-mounted dissecting microscope at 60× power using Kodak 160T film (Eastman Kodak Company, Rochester, NY, USA).

Annexin V and TUNEL analysis

Propidium iodide and annexin V staining of living fragmented embryos were performed as described by Van Blerkom and Davis (1998) in order to determine cell membrane integrity and presence of phosphatidylserine residues on the outer surface of the plasma membrane, respectively. Embryos were first incubated at 37°C for 45 min in PBS containing propidium iodide (500 ng/ml), examined by fluorescence microscopy, followed by incubation in the presence of an FITC conjugate of annexin V (1 μg/ml) according to the manufacturer's recommendation (ApoAlert Annexin V Apoptosis System; Clontech, Palo Alto, CA, USA). These analyses were performed at 37°C in a ΔT system (Bioptechs, Butler, PA, USA) as described previously (Van Blerkom et al., 1995a). In preparation for TUNEL, after annexin/propidium analysis, embryos were fixed in 3.7% formaldehyde in PBS (pH 7.35), washed for at least 12 h in PBS containing 1% BSA and incubated for 4 h at room temperature in TBS (155 mM NaCl, 10 mM Tris–HCl pH 7.4) containing 0.1% NP-40 and 0.1% Triton X-100. Within 24 h of fixation, embryos were washed three times in PBS–1% BSA and incubated at 37°C for 1 h in TUNEL reaction mixture containing 0.5 U/μl of calf thymus terminal deoxynucleotidyl transferase and a fluorescein-labelled nucleotide mixture (F-dUTP) (In Situ Cell Death Detection System; Boehringer Mannheim, Indianapolis, IN, USA) as described previously (Van Blerkom and Davis, 1998). For fluorescent analysis of fixed embryos, specimens were placed into a droplet of Slow Fade Lite (Molecular Probes, Eugene, OR, USA) and examined by conventional and scanning laser confocal microscopy. For the examination of DNA integrity on embryos that had been previously analysed for protein domains by immunofluorescence, these specimens were resuspended in PBS–1% BSA to rehydrate, incubated in TUNEL reagents and re-examined by scanning laser confocal microscopy. TUNEL-negative embryos were secondarily used as positive controls by exposing them to DNase II for reincubation in TUNEL reagents as previously described (Van Blerkom and Davis, 1998). TUNEL-positive embryos were stained with 4,6-diamidino-2-phenylindole diacetate (DAPI; 50 μg/ml) and re-examined by scanning laser confocal and conventional microscopy, respectively, to demonstrate that TUNEL and nuclear fluorescence were coincident.

Western blot analysis

The presence of leptin and STAT3 in human oocytes and embryos was confirmed previously (Antczak and Van Blerkom, 1997), and other studies have shown that TGFβ2, c-kit and EGF-R proteins are present in mammalian oocytes and embryos, including human (see below). The Bax, Bcl-x and VEGF primary antibodies used during these analyses were reactive for both mouse and human proteins, as confirmed here by the detection of polarized domains for each of these proteins in immunostained MII stage mouse oocytes, following scanning laser confocal microscopic examination as described above for human samples. To further substantiate the presence of Bax, Bcl-x and VEGF proteins in oocytes and embryos, mouse oocytes were examined by Western analysis. Similar Western blot analyses with human oocytes were not possible. For the examination of each of

these proteins, a lysate derived from 600–800 mouse oocytes was applied to each lane. Mouse oocytes were recovered from the ovaries of naturally cycling ICR mice, denuded of granulosa and coronal cells, and processed for Western blot analysis as previously described (Antczak and Van Blerkom, 1997). For the analysis of Bax and Bcl-x proteins, oocytes were lysed in a solution containing 125 mM Tris–Cl pH 6.8, 20% glycerol, 4% SDS, 286 mM 2-mercaptoethanol (2-BME) and 10 µg/ml bromophenol blue and boiled for 15 min before loading onto a 10% SDS–PAGE gel for electrophoretic separation. To serve as a standard for both the Bax and Bcl-x proteins, a small aliquot of a 3T3 RSV-transformed cell lysate (Transduction Laboratories, Lexington, KT, USA) was processed as per oocyte lysates and run in lanes adjacent to those containing oocyte lysates. For the analysis of VEGF, oocytes were lysed in a solution containing 125 mM Tris–Cl pH 6.8, 20% glycerol, 4% SDS, 10 µg/ml bromophenol blue and with (reducing) and without (non-reducing) 286 mM 2-BME. Both reduced and non-reduced lysates were boiled for 15 min before loading onto a 12% SDS–PAGE gel for electrophoretic separation. To serve as a standard for VEGF, an aliquot of purified protein (293-VE-010; R&D Systems, Minneapolis, MN, USA) was processed in an identical manner and run in lanes adjacent to those containing oocyte lysates. Following electrophoresis, proteins were electroblotted to polyvinylidine difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) in preparation for antibody analysis. Bax proteins were detected using a 1:1000 dilution of a rabbit anti-Bax polyclonal antibody (I-19; Santa Cruz Biotechnology Inc.) and Bcl-x proteins were identified using a 1:1000 dilution of a rabbit anti-Bcl-x polyclonal antibody (S-18; Santa Cruz Biotechnology Inc.) in conjunction with an amplified alkaline phosphatase immunoblot assay kit (Bio-Rad) following the protocols provided by the manufacturer. To confirm that the detected proteins were specific to the antibodies used, Bax and Bcl-x primary antibodies were preincubated with a 20-fold excess of blocking peptide (SC-526P and SC-643P, respectively; Santa Cruz Biotechnology Inc.) for 2.5 h at 37°C before their use for immunodetection of electrophoresed 3T3-RSV lysates. Under these conditions no proteins were detected. VEGF proteins were identified using two independent mouse anti-VEGF monoclonal antibodies. One of these antibodies (C1; Santa Cruz Biotechnology Inc.) was used at a 1:400 dilution to detect VEGF in standard and oocyte lysates electrophoresed under reducing conditions. The second monoclonal antibody (MAB293; R&D Systems) was used at a 1:500 dilution to detect VEGF in standard and oocyte lysates electrophoresed under non-reducing conditions. Primary antibody reactivity was identified using a 1:500 dilution of a goat anti-mouse streptavidin conjugate (401213; Calbiochem-Novabiochem Corp., San Diego, CA, USA) in conjunction with an amplified alkaline phosphatase immunoblot assay kit (Bio-Rad) following the protocols provided by the manufacturer.

Results

Temporal and spatial aspects of fragmentation in cleavage stage human embryos

The in-vitro development of 2293 fertilized eggs from 257 IVF cycles was monitored at 8- to 12-h intervals during at least 3 days of culture. Some form of fragmentation was clearly evident at the level of the dissecting microscope in approximately 41% (934/2293) of the embryos, and the approximate time and pattern of fragmentation were documented at 6- to 8-h intervals for 607 representative embryos obtained during 66 conventional IVF cycles. The stages of development at which fragmentation first occurred in these

Table I. Stage at which fragmentation was first detected in 607 cultured human embryos produced by in-vitro fertilization

Stage	No of embryos
1-cell	153
2-cell[a]	211
2-cell[b]	29
4- to 6-cell	146
6- to 8-cell	68

[a]Fragmentation involved one blastomere.
[b]Fragmentation involved both blastomeres.

607 embryos are summarized in Table I. In contrast, none of 297 MII oocytes destined for ICSI or 109 excess MII oocytes donated from GIFT procedures (denuded of cumulus and coronal cells within 2 h after follicular aspiration) showed any signs of fragmentation when examined at high magnification with phase and differential interference contrast optics. A type 2 fragmentation pattern (see below) was observed in four of 562 (<1%) unfertilized and unpenetrated MII oocytes that were examined over 2–3 days of culture. In each case, fragmentation was not observed until after approximately 48 h of culture.

The following four basic patterns of fragmentation (types 1–4) were observed in this study: (i) the presence of a monolayer-like carpet of very small fragments that coated only a 'modest' portion of the cell surface with either no apparent reduction in cell size, or one which was relatively minor but evident at the light microscope level; (ii) the presence of multiple layers of fragments, generally involving a considerable portion of the cell surface, which was accompanied by a significant reduction in the size of the affected cell or its subsequent complete disintegration; (iii) the complete disintegration of a blastomere(s) in a cleavage stage embryo where no indications of extracellular fragment formation were detected on previous examinations; and (iv) a very limited number of small fragments scattered over several blastomeres in otherwise normal-appearing, developmentally progressive embryos. Spatial and temporal characteristics of each fragmentation pattern and the apparent developmental consequences for the affected embryo of types 1–4 fragmentation are discussed in detail below.

Fragmentation at the pronuclear and 2-cell stages

Fragmentation at the 1-cell stage was detected in approximately 7% (153/2293) of pronuclear and syngamic eggs during routine inspection. Retrospective analyses of recorded images of 91 embryos taken from the pronuclear through the cleavage stages demonstrated that fragments, which were evident at the 2-cell stage, actually arose during the pronuclear stage. The representative images in Figure 1K and L show extracellular fragments (asterisks) in the region of the first and second polar bodies in the same egg at the pronuclear and syngamic stages, respectively. The same fragments appeared to be present at the 2-cell stage (Figure 1M), and in similarly fragmented embryos where cytokinesis continued they frequently appeared to remain unchanged through the cleavage stages. During routine inspections of fertilized eggs at the level of the
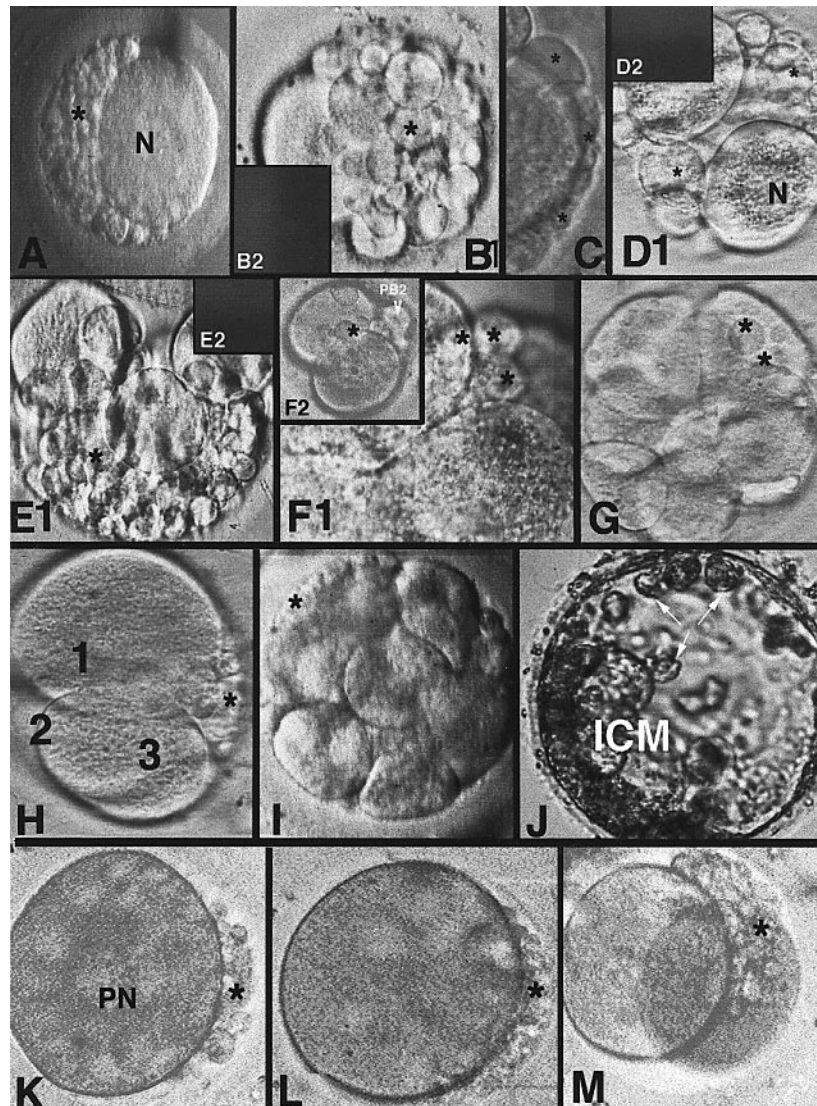
Figure 1. Light microscopic images of living human embryos at the 1-cell (**A**, **K**, **L**), 2-cell (**B1**, **C**, **M**), 4- to 14-cell (**D1**–**I**) and blastocyst (**J**) stages; four different patterns of fragmentation are presented in this figure. Type 1 (C, F1–2, H, I, J); type 2 (A, B1); type 3 (D1, E1); and type 4 (G) patterns of fragmentation are described in the text. (F2, K–M) Images captured from videotape. (B2, D2, E2) Epifluorescent images of the corresponding embryo stained with annexin V. (H–J) Images of the same embryo taken at the 3-cell (H, blastomeres 1–3 are evident in this view), 12–14-cell (I) and expanded blastocyst stages (J), where acellular fragments in proximity to the inner cell mass (ICM) are indicated by arrows. In (A–I) and (K, L) fragments are indicated by asterisks. (See text for details.) Original magnification: (A) ×350; (B1) ×400; (F1) ×850; (G) ×675; (K–M) ×550.

dissecting microscope, relatively small fragments of this type may not be evident or may not be noted until later in development, unless the egg or embryo is completely rotated during each examination period. For example, the fragments noted during the detailed inspection of an embryo at the 4-cell stage (Figure 1, panel F1) were of the same size, number and general location as those on the same embryo at the 2-cell stage when images obtained during an earlier, brief examination were reviewed (Figure 1, panel F2). Here, type 1 fragmentation refers to a distinct pattern of fragmentation characterized by the formation of a carpet of small fragments that were generally of uniform size and which arose from a portion of the plasma membrane, primarily during the 1-cell stage (Figure 1, panels K–M). Typically, eggs or blastomeres displaying type 1 fragmentation remained intact. Some 73% of fragmented 1-cell eggs (111/153) displayed this pattern of

which 87% (97/111) underwent at least one cell division (see below).

An elaboration of the type 1 pattern of fragmentation involving a greater portion of the plasma membrane was observed in pronuclear eggs (27%; 42/153) as well as in cleavage stage embryos (e.g. Figure 1C). Here, this pattern of fragmentation is termed type 2 and was characterized by the generation of a relatively large population of fragments from a significant portion of the oolemma or blastomere plasma membrane with a corresponding reduction in the volume of the affected egg or blastomere(s). In the present study, the occurrence of type 2 fragmentation at the 1-cell stage resulted in development arrest for all affected embryos either at the pronuclear (88%; 37/42; Figure 1A) or syngamic stages (12%; 5/42). Scanning laser confocal microscopic analysis of the type 2 pattern of fragmentation suggested that these extrusions

arose from the surface of the cell in a column-like manner (Figure 2P and S3; Figure 3I1, L and M), and in some instances, smaller fragments appeared to be generated by second-order membrane extrusions from pre-existing, larger fragments (Figure 2P and Figure 3L and M, black asterisks). In serial optical sections, an apparent continuity between the cortical ooplasm and the interior of fragments adjacent to the oolemma was frequently observed (Figure 2P, white asterisks).

For 240 embryos which fragmented at the 2-cell stage and were examined in detail, most instances of fragmentation appeared to involve one blastomere (88%, $n = 211/240$) and the type 2 pattern. For 62% of these embryos ($n = 131/211$), the complete disintegration of a blastomere (Figure 1B1, black asterisk) was preceded by the elaboration of numerous, small spherical structures which arose from the plasma membrane either during the perisyngamic stage or shortly after completion of the first cell division (Figures 1C and 2E, black asterisks). In contrast, the fragmentation pattern of 38% of the embryos within this group which fragmented at the 2-cell stage ($n = 80/211$) was characterized by the conversion of a single blastomere into several large cytoplasts (Figure 2H and I). In these instances, the complete fragmentation of a blastomere in a previously normal-appearing embryo occurred rapidly and was not seen to be preceded by the generation of extracellular fragments detectable during earlier examinations; we have termed this pattern of blastomere disintegration type 3. We cannot exclude the possibility that type 1 or 2 fragmentation can occur rapidly and serve to initiate this more disruptive activity (type 3). Fragmentation involving both blastomeres occurred in 12% (29/240) of the embryos examined in detail in this study. In some embryos, both blastomeres were disrupted completely ($n = 7$; type 3) while in others ($n = 22$) one blastomere showed a type 2 pattern and the other was classified as type 3. No further cell division occurred in any of these type 2/type 3 fragmented embryos. Some 38% of 2-cell embryos in which a single blastomere fragmented into multiple, relatively large cytoplasts (type 3; $n = 80/211$) showed no further development, while 62% of 2-cell embryos in which a single blastomere fragmented (131/211) involving type 2 fragmentation underwent at least one additional cell division (Figure 1D1), with 47% of these ($n = 61/129$) developing to the 6-cell stage when embryo transfers were performed (Figure 1E1). However, no estimation of the implantation potential of embryos that continued to divide after fragmentation at the 2-cell stage could be derived from nine pregnancies that occurred in 24 cycles where at least one such 6-cell embryo was included along with unfragmented embryos in cohorts of three to four transferred embryos.

Among all 2-cell embryos, 9% ($n = 178/1986$) that appeared normal at the dissecting microscope level were found to contain a small cluster of type 1 fragments associated with one or both blastomeres [e.g. Figure 2B and F (arrow) when viewed at higher magnifications (200–400×)]. These structures were not detected at the pronuclear stage and usually resided either in the cleft between blastomeres or on the surface at one pole of a single blastomere. However, the complete fragmentation of an affected blastomere(s) was not observed during subsequent culture, although an apparent reduction in the size of a

blastomere was not unusual (e.g. right blastomere, Figure 2B). Most of these embryos continued to divide (79%; 141/178) through the cleavage stages (portion of a fragment cluster is indicated by an asterisk in Figure 1H) and at the morula stage these fragments were clustered on one portion of the embryo surface (Figure 1I, asterisks). Thirty-one of these embryos were included in 27 cohorts of transferred embryos that also contained normal-appearing, unfragmented 8- to 14-cell embryos (transfer day 3.0 or 3.5). Twelve pregnancies occurred of which 11 were ongoing and resulted in 17 babies. Whether any of the fragmented embryos contributed to these births could not be determined. However, 23 embryos with this phenotype were retained in culture for a total of 6 days. Of the eight that developed to the expanding blastocyst stage (35%), only one was characterized as normal (Van Blerkom, 1993). Most blastocysts derived from these embryos, as well as those that developed to the blastocyst when type 1 fragmentation occurred at the 1-cell stage ($n = 16$), appeared growth-retarded with respect to cell number, contained cells and cellular fragments free in the blastocoel, and exhibited a disorganized or malformed inner cell mass (Figure 1J). The fragments indicated by arrows in Figure 1J showed no staining with DNA-fluorescent probes and were classified as acellular.

Fragmentation at the 4- to 8-cell stage

For 146 embryos, the presence of a polymorphic cluster(s) of fragments was first detected at the 4- to 6-cell stage (Figure 2H and I). No evidence of fragment formation was observed during earlier inspections and the fragments appeared to be associated with the complete disintegration of a single blastomere (type 3 pattern). Some 72% (105/146) of these embryos continued to divide during the subsequent 22–24 h of culture. For 16 patients, all of the embryos transferred (3–4/patient, $n = 57$) displayed this phenotype, and five term pregnancies (31%) and eight babies resulted. This finding demonstrates that at least 14% (8/57) of this type of fragmented embryo were developmentally viable. Likewise, a similar cluster(s) of type 3 fragments was first detected at the 6- to 8-cell stage in 68 embryos. All 32 of the 8- to 10-cell embryos transferred to 10 patients on day 3 (72–78 h, 3–4/patient) contained a clear complement of numerous extracellular fragments that had developed approximately 24 h earlier. Four pregnancies resulted in six babies, demonstrating that at least 19% of these fragmented embryos were developmentally viable. Thirty-eight embryos with type 3 fragmentation were retained in culture for 6 days and 50% (19/38) developed to the hatched blastocyst stage in an apparently normal manner (Van Blerkom, 1993). The precise sequence of events leading to fragmentation detected at the later stages could not be determined.

Fragmentation in grossly normal-appearing cleavage stage embryos

Approximately 1750 embryos examined in this study appeared grossly normal and developmentally progressive during 2–3 days of culture. However, when inspected at high magnification with phase or differential interference contrast optics just before uterine transfer, approximately 68% (1185/1743) were
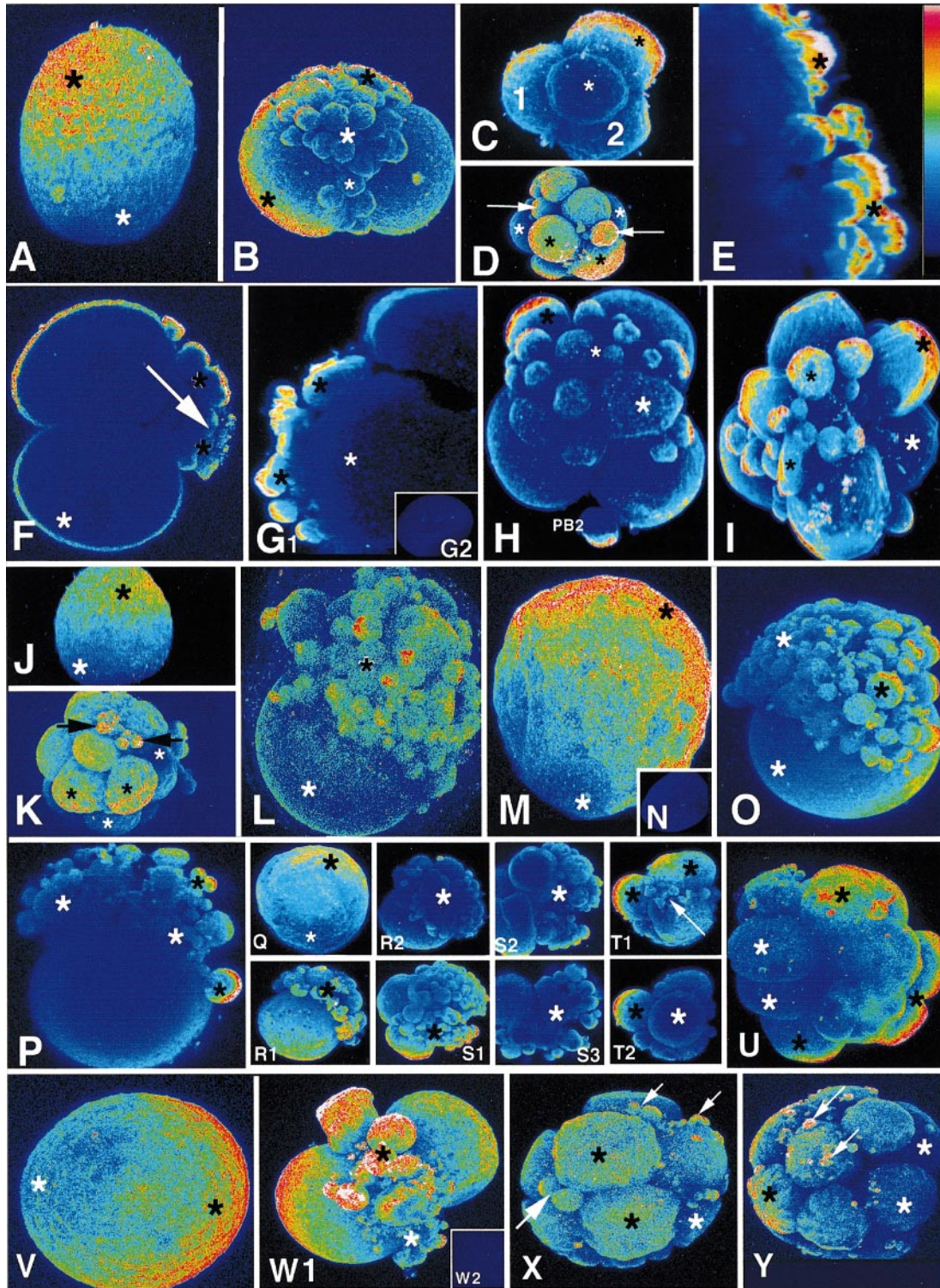
Figure 2. Scanning laser confocal immunofluorescence, pseudo-colour images demonstrating the polarized domains of leptin (**A–I**), STAT3 (**J–L**), Bcl-x (**M–P**), Bax (**Q–U**) and TGFβ2 (**V–Y**) in normal human oocytes (A, J, M, Q, V) and cleavage stage embryos (C, D, K, T1, T2, U, X, Y), and the disruption of those domains in fragmented embryos (B, E–G1, H, I, L, O, P, R1–2, S1–3, W). Regions containing protein-rich domains in oocytes, blastomeres and fragments are indicated with black asterisks; regions largely devoid of these proteins are indicated with a white asterisk. Panels (A–D, G2, H–O, Q, R1, S1, U–Y) are fully compiled images (entire specimens). Panels (E–G1, P, R2, S2–3, T1–2) are partially compiled images of internal sections. Panels (B, F, O, P, R1–2, S1–3, T1–2) show different portions of the same embryo. Examples of type 1 (B), type 2 (O, P, S1–3), type 3 (H, I) and type 4 (D, K, T1–2, X, Y) fragmentation patterns are shown as indicated. Representative oocytes immunostained in the presence of blocking peptides for leptin, Bcl-x, and TGFβ2 are shown in panels G2, N and W2, respectively. The relative intensity of immunofluorescence is indicated in the colour bar in (E). (See text for details.) Original magnification: (A) ×300; (C) ×250; (E) ×850; (J,Q) ×200; (M,V) ×400.
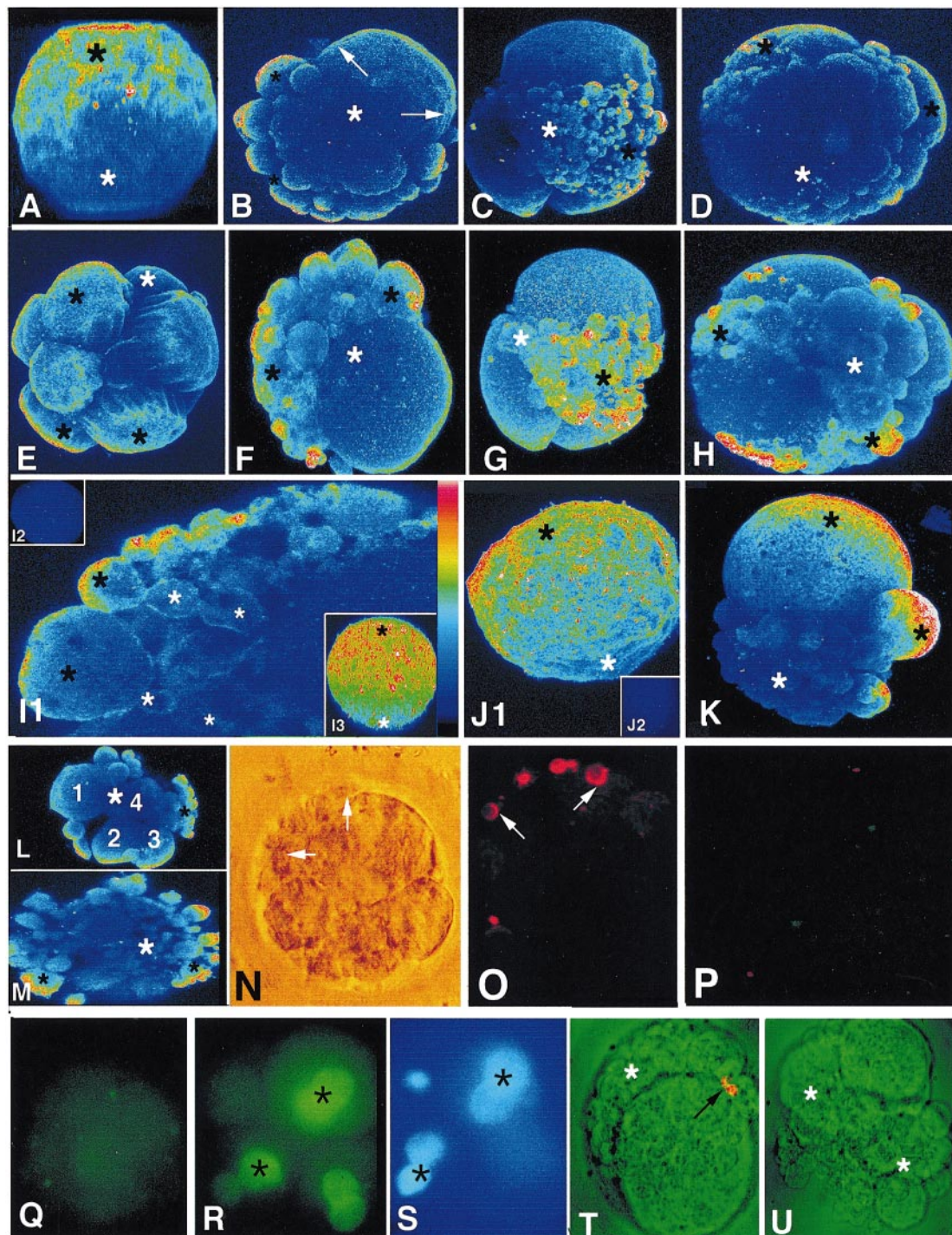
Figure 3. Scanning laser confocal immunofluorescence, pseudo-colour images demonstrating polarized domains of VEGF (**A–E**), c-kit (**F–I**) and EGF-R (**J–M**) in normal human oocytes (A, I3, J1) and cleavage stage embryos (E), and the disruption of those domains in fragmented embryos (B–D, F–I1, K–M). Regions containing protein-rich domains in oocytes, blastomeres and fragments are indicated with black asterisks; regions largely devoid of these proteins are indicated by white asterisks (A–M). Panels (A–H, I2–K) are fully compiled confocal images. Panels (**N, O–Q**) are phase contrast and epifluorescent images, respectively. Panels (I1, L, M, R, S) are partially compiled images of internal sections. Panels (L, M) show different portions of the same embryo. Panels (B, F, C, G, D, H) show embryos dual-stained for VEGF and c-kit, respectively. Panels (**N–S**) are representative images of a type 2 fragmented 8-cell embryo observed by light microscopy (N) and by conventional epifluorescence microscopy in the living state after staining with propidium iodide (O) and annexin V (P). After fixation, this representative embryo was examined by TUNEL before (Q) and after DNase treatment (R) and staining with DAPI (S). Panels (**T, U**) are phase contrast images of fragmented embryos upon which corresponding TUNEL results obtained by conventional epifluorescence microscopy were superimposed [black arrow = positive TUNEL signal; (T)]. Extracellular fragments are indicated by white arrows in (N, O) and by white asterisks in (T, U). (T, U) correspond to Figure 2R–S. Fully compiled scanning laser confocal microscopic images (i.e. the entire oocyte) of representative oocytes immunostained in the presence of blocking peptides for c-kit and EGF-R are shown in (I2) and (J2), respectively. The relative intensity of immunofluorescence is indicated in the colour bar in (I1). (See text for details.) Original magnification: (A–E) ×300; (I1) ×900; (N) ×400.

found to contain a relatively small number (typically 6–10) of spherical structures on the surface of one or more blastomeres (Figure 1G, asterisks; Figure 2K, black arrows; Figure 2T1, X and Y, white arrows). Here, we have characterized this pattern of fragmentation as type 4. While the stage at which these 'fragments' formed was unclear, their size and distribution suggested that they were not remnants of the first polar body. When representative embryos donated for research were examined with DNA-fluorescent probes ($n = 16$), no chromosomal staining was observed in the fragments (data not shown). In those cases where affected embryos were viewed on edge, these extracellular structures were separated from the underlying plasma membrane and therefore unlikely to include the second polar body (Gardner, 1997). Outcomes from 116 transfers in which all of the 6- to 12-cell embryos were classified as type 4, or were free of detectable extracellular fragments, suggested that the type 4 fragmentation pattern had no significant influence on developmental potential. Fifty-three transfers in which all embryos ($n = 186$) were classified as type 4 resulted in 24 ongoing pregnancies (pregnancy rate = 45%) and 32 babies (implantation rate = 17%). Sixty-three embryo transfers in which all embryos ($n = 214$) were free of detectable extracellular fragments resulted in 27 ongoing pregnancies (pregnancy rate = 43%) and 39 babies (implantation rate = 18%). There were no differences between these two groups with respect to the incidence of biochemical or failed clinical pregnancies.

The occurrence of polarized domains of regulatory proteins in human oocytes and fragmented embryos

In addition to leptin (Figure 2A) (unfertilized or uninseminated and unpenetrated oocytes; O, $n = 32$) and STAT3 (Figure 2J) (O; $n = 13$) whose polarized distribution in the MII oocyte and pronuclear egg was described previously (Antczak and Van Blerkom, 1997), scanning laser confocal immuno-fluorescence demonstrated that the distribution of Bcl-x (Figure 2M; O, $n = 18$), Bax (Figure 2Q; O, $n = 18$), TGFβ2 (Figure 2V; O, $n = 9$), VEGF (Figure 3A; O, $n = 22$), c-kit (Figure 3I3; O, $n = 7$) and EGF-R (Figure 3J1; O, $n = 25$) were also localized in polarized, subplasmalemmal domains in the mature oocyte. Multiple examinations of representative specimens, including those inverted 180° between scans, demonstrated that the location of the domains was independent of the position or orientation of the specimen during scanning laser confocal imaging. Similar to the earlier finding that leptin and STAT3 domains are unequally partitioned to daughter blastomeres during early cleavage (leptin, Figure 2C and D; embryos, E, mostly fragmented, $n = 32$), STAT3 (Figure 2K; E, $n = 6$), an unequal distribution of the other proteins examined in this study was also evident in early cleavage stage embryos (e.g. Bax, Figure 2T1 and T2; E, $n = 8$); TGFβ2 (Figure 2X and Y; E, $n = 8$); and VEGF (Figure 3E; E, $n = 14$). For embryos that fragmented between the 2- and 4-cell stages, the normal distribution of the polarized protein domains detectable by immunofluorescence was related to the pattern of fragmentation and the region of the blasto-mere(s) from which the fragments emerged. For example, in the anti-leptin-stained 2-cell embryo shown in Figure 2B,

numerous small spherical structures (white asterisks) were present in the cleft between the two blastomeres in this representation of type 1 fragmentation. Owing to the smaller size of the blastomere on the right-hand side and the near-total depletion of its polarized domain (Figure 2B), it is assumed that most of these fragments originated from this cell. Of particular note are the polarized distribution of leptin immunofluorescence among the fragments and the relative abundance of leptin in the largely intact blastomere on the left (Figure 2B, black asterisk). This is in contrast to the typical pattern of leptin distribution in a normal 2-cell embryo where both blastomeres would be expected to contain polarized domains, with one blastomere staining slightly more intensely than the other (Figure 4, M–P) (Antczak and Van Blerkom, 1997). In the case of the embryo depicted in Figure 2B, the pattern of anti-leptin staining associated with the fragments followed the expected distribution of the leptin domain within the blastomere before fragmentation, such that a fragment-borne gradient of immunofluorescence was observed with bright staining at one end of the embryo (Figure 2B, black asterisk at top) and reduced staining at the other (white asterisks). A similar effect of fragmentation on cortically located polarized protein domains was also observed for STAT3 (Figure 2L), Bcl-x (Figure 2O; E, $n = 10$), Bax (Figure 2R1 and R2), TGFβ2 (Figure 2W1), VEGF (Figure 3C), c-kit (Figure 3G; E, $n = 7$) and EGF-R (Figure 3K; E, $n = 7$). For all of the proteins examined in this study, when fragmentation involved the portion of a blastomere that did not contain the protein domains, only background levels of the immuno-fluorescent signal were detected in the fragments (e.g. VEGF; Figure 3D, white asterisk; EGF-R; Figure 3K, white asterisk).

During type 2 fragmentation, characterized by the presence of columns of contiguous fragments, the outermost fragments displayed the highest immunofluorescent signal for each of the proteins examined, and the strength of the associated immunostaining dropped precipitously among the underlying members as proximity to the cell surface approached (e.g. Bcl-x, Figure 2P, asterisks; Bax, Figure 2S1 and S2, asterisk; c-kit, Figure 3I1, asterisks; EGF-R, Figure 3L and M). In cases where fragmentation involved the elaboration of small, spherical structures that appeared to 'bud' from the cell surface, the near-complete (e.g. leptin, Figure 2G, asterisks) or partial (e.g. leptin, Figure 2E and F, asterisks) elimination of the polarized domain from the affected blastomere was observed.

Thirty-six embryos appeared grossly normal at the 2- and early 4-cell stages, but fragmentation of a single blastomere was evident when these embryos were re-examined 12 h later (Figure 2H and I). Twenty-three of these embryos divided to the 6- to 8-cell stage, but only four progressed to the morula stage. The disappearance of a blastomere from a 4- to 6-cell embryo typically involved its fragmentation into a pleio-morphic population of relatively large cytoplasts (type 3) rather than the elaboration of numerous, small structures such as observed in some 2-cell embryos (types 1 and 2). The absence of a detectable immunofluorescent signal in virtually all fragments was observed only in rare cases. In these instances, it appears likely that the affected cell was the one that inherited only a portion of the polarized domain during the

previous cell division (e.g. Figure 2C, cell 1 or 2), or was largely devoid of a domain for the particular protein examined (Antczak and Van Blerkom, 1997). Examples of such a cell(s) are present in an anti-leptin-stained 4-cell embryo (Figure 2C, white asterisk), an 8-cell embryo (Figure 2D, white asterisks), a 10-cell embryo stained with anti-Bax antibody (Figure 2U, white asterisks) and an 8-cell embryo stained with anti-VEGF (Figure 3E, white asterisk). In each of these figures, a black asterisk indicates examples of blastomeres containing polarized protein domains. Most cytoplasmic fragments stained positively for the proteins examined in this study in nearly all embryos in which blastomere fragmentation occurred after the 4-cell stage (anti-leptin, Figure 2I; anti-Bax, Figure 2S1; anti-

EGF-R, Figure 3L and M). Regardless of the pattern/type of fragmentation, the most intense immunofluorescent signal for each of the eight proteins examined was invariably localized to the apical portion of the fragments (e.g. asterisks, Figure 2E–I, O, P and S1–S3; Figure 3B, C, F, H, I1 and K–M). This distribution was both characteristic of the cytoplasts and indicative of the pronounced subplasmalemmal localization of the polarized protein domains observed in the intact blastomere(s) (Figure 2C, D and U; Figure 3E). While fragmentation of whole blastomeres was evident at the level of the dissecting microscope, it is important to note that even for those 6- to 8-cell embryos which appeared normal at high magnification, in many cases some extracellular fragments could be identified
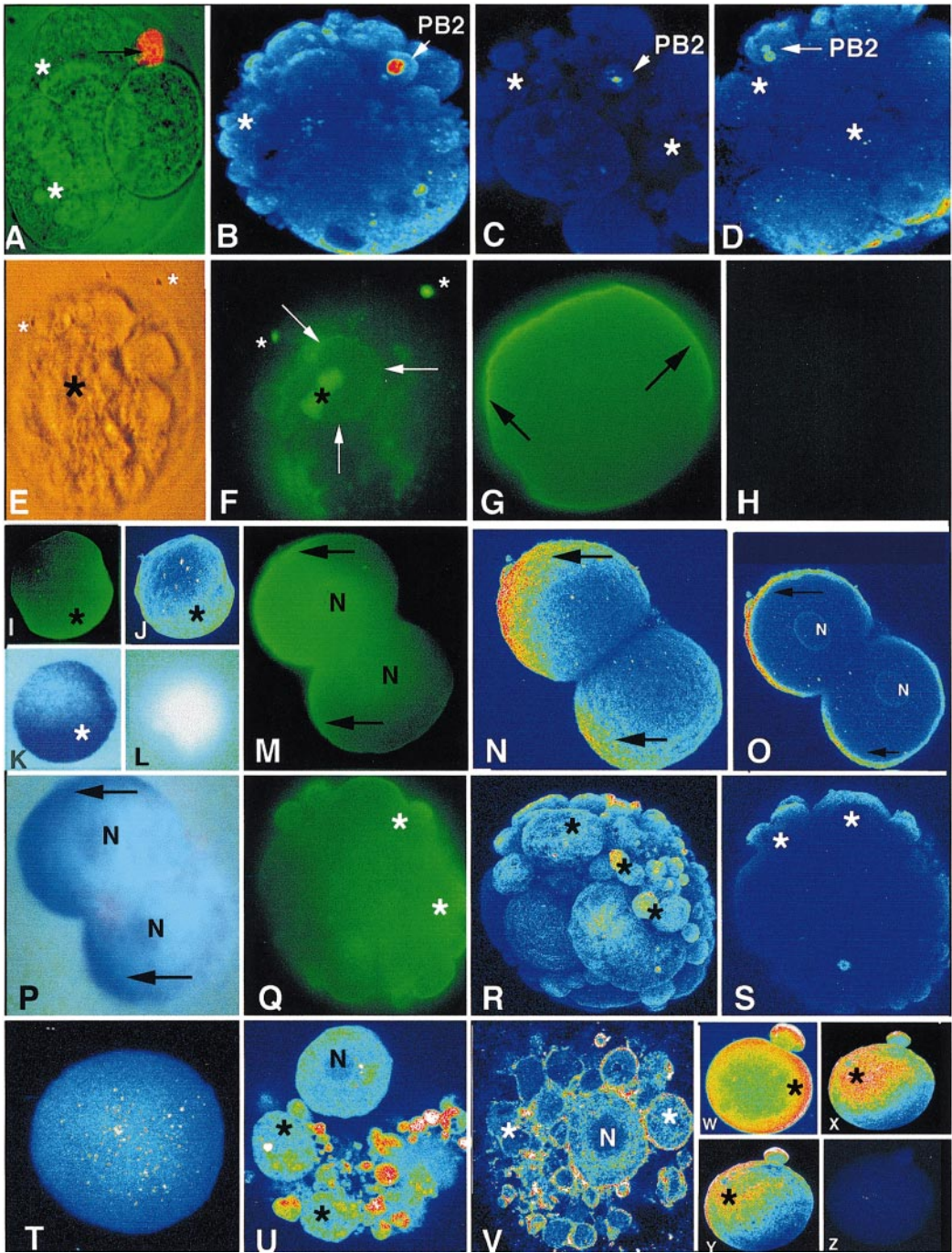
upon closer examination (e.g. arrows in Figure 2D, K, T1, X and Y). With respect to fragment-associated immunofluorescence, the primary determinant of the presence or absence of a signal for the proteins examined in this study was whether or not an immunofluorescent signal occurred in the subjacent subplasmalemmal cytoplasm of the blastomere in the precise location from which the fragment(s) arose.

To confirm the specificity of the antibodies used during this study, unpenetrated and uninseminated MII oocytes were stained with each of these antibodies following the preincubation of each with an excess of the peptides used to generate the antibody, with the exception of the antibody directed against VEGF where blocking peptide was unavailable. Figure 2G2, N and W2 and Figure 3I2 and J2 are representative fully compiled images of entire oocytes stained with particular antibodies following their preincubation with corresponding blocking peptides. For the seven proteins analysed in this manner, no appreciable immunofluorescence was detected. To serve as controls during single and dual antibody analyses, human oocytes ($n = 6$) and embryos ($n = 9$) were stained with non-specific primary and secondary antibodies, and no immunofluorescence was detected (Figure 4H) except as noted below.

E-cadherin and actin immunostaining

As an internal control for a cytoplasmic protein which is uniformly distributed in human oocytes and early embryos (Campbell *et al.*, 1995), oocytes (O) and normal and fragmented embryos (E) were immunostained for E-cadherin (uvomorulin) (O, $n = 8$; E, $n = 5$). Scanning laser confocal microscopic analysis of uvomorulin-stained samples demonstrated uniform fluorescence throughout the cytoplasm of the oocyte (Figure 4T), the blastomeres of early normal (approximately 2- to 6-cell) and fragmented embryos and their cytoplasmic fragments (Figure 4U). As an internal control for a cytoplasmic protein that is localized primarily in the cytocortex, normal

oocytes and fragmented human embryos were stained with an anti-actin antibody (O, $n = 14$; E, $n = 3$). Analysis of actin-associated immunofluorescence by scanning laser confocal microscopy revealed the most intense immunofluorescence localized to the cortex of the oocyte and individual blastomeres and in the subplasmalemmal cytoplasm of various-sized fragments (Figure 4V).

Detection of protein domains by sequential fluorescent and colorimetric analysis

In order to demonstrate that the presence and distribution of proteins in polarized domains was not related to the method of immunodetection, human oocytes and embryos were examined by different immunoanalytical methods. For example, human oocytes ($n = 6$), normal embryos ($n = 4$) and fragmented embryos ($n = 3$) in which leptin was detected initially by immunofluorescence were reanalysed for antibody reactivity using a colorimetric detection system. Following immunofluorescent and colorimetric detection of primary antibody reactivity, conventional epifluorescent, scanning laser confocal microscopic and colorimetric images of each sample were compared for the presence and distribution of the respective protein as represented by each approach. As shown for a representative unfertilized oocyte (Figure 4I–L), 2-cell embryo (Figure 4M–P) and fragmented embryo (Figure 4Q–S) immunostained for leptin, there was close agreement on the presence and distribution of this protein detected in individual specimens by conventional epifluorescence microscopy (Figure 4I, M and Q), scanning laser confocal microscopy (Figure 4J, N, O, R and S) and colorimetric analysis (Figure 4K and P). This close agreement was true even though the three approaches do not precisely convey the same types of information. For example, single epifluorescent images cannot adequately present information regarding unique distributions of fluorescence throughout an entire human oocyte or embryo. Epifluorescent images represent the result obtained at a given plane

Figure 4. Combined light and fluorescence image of a representative fragmented embryo analysed by TUNEL before immunostaining (Figure 2T). The arrow in (**A**) indicates TUNEL-positive fluorescence associated with the putative second polar body. (**B, D**) Scanning laser confocal immunofluorescence, pseudo-colour images of fully compiled embryos previously stained for polarized protein domains (note the bleed-through of the fluorescent signal) and subsequently examined for apoptosis by TUNEL. (**C**) Fully compiled image of representative type 3 fragmented 8-cell human embryo (asterisks = fragments) examined by scanning laser confocal microscopy after TUNEL staining. (**D**) Type 3 fragmented 8-cell embryo. (**E, F**) Light and epifluorescence images, respectively, of a type 3 fragmented embryo in which a single nucleus showed positive TUNEL fluorescence (black asterisk). The relative translucency of the cytoplasm made this particular blastomere difficult to detect in the light microscope (**E**, black asterisk), but under epifluorescence illumination it was observed to be of normal size and not surrounded by fragments (**F**, white arrows). The only other intense TUNEL fluorescence detected in fragmented embryos of this type was associated with residual spermatozoa (E,F, white asterisks). (**G**) A photograph of the epifluorescence signal from an unfertilized human oocyte stained for leptin; the subplasmalemmal polarized domain is evident (black arrows). A corresponding representative control for the specimen in (**G**) is shown in (**H**); settings and exposure times were identical to those used for (**G**). (**I, J**) Leptin domain (black asterisk) in an unfertilized human oocyte stained for leptin as detected using epifluorescence and the scanning laser confocal microscope, respectively. (**K**) Leptin domain (white asterisk) of the same specimen as in (I,J) as identified by colorimetric immunodetection. A corresponding colorimetric control for the analysis of the sample in (**K**) is shown in (**L**). (**M–P**) Leptin domains = black arrows; N = nuclei); (**Q–S**) White or black asterisks indicate regions of elevated leptin immunofluorescence. Panels show images of the same 2-cell and fragmented embryo, respectively, stained for the leptin protein using: (i) immunofluorescence as detected by epifluorescent analysis (**M,Q**) or scanning laser confocal microscopy (**N, O, R, S**); and (ii) colorimetric immunodetection (**P**). (**T**) An unfertilized human oocyte stained for E-cadherin; punctate points of intense fluorescence are background signals of unknown origin. (**U, V**) Internal portions of fragmented human embryos stained for E-cadherin and actin, respectively. (**U**) N = nucleus; black asterisks = fragmented blastomere. (**V**) N = nucleus; white asterisks = fragmented blastomere. (**W**) An MII stage murine oocyte stained for Bax (black asterisk = polarized domain). (**X, Y**) An MII stage murine oocyte dual-stained for Bcl-x and VEGF (black asterisk = polarized domain), respectively. Results from a representative MII stage oocyte which served as control during these dual-staining analyses is shown in (**Z**). Original magnification: (**A**) ×350, (**E**) ×380; (**I**) ×150; (**M**) ×350; (**Q**) ×380; (**W–Z**) ×200.

of focus and the fluorescent signal is influenced by fluorescence above and below this plane. Scanning laser confocal microscopic images can accurately portray both fluorescence throughout an entire specimen as well as at any specific portion of the specimen without contaminating fluorescence from above and below the plane(s) of interest. For these reasons, confocal images displaying both internal portions of a normal 2-cell (Figure 4O) and fragmented 4-cell embryo (Figure 4S), as well as those images showing fluorescence throughout the entire specimens are shown (Figure 4N and R, respectively). These images should be compared with corresponding images derived by conventional epifluorescent (Figure 4M and Q) and colorimetric analyses (Figure 4P). Unfertilized oocytes used as controls for colorimetric analysis showed no colour development (Figure 4L).

None of the samples examined in this study showed any subplasmalemmal or cortical autofluorescence. Occasionally, relatively intense, random points of autofluorescence which may be associated with lipid droplets were observed in the cytoplasm of some unpenetrated oocytes (Figure 4T), but not in normal or fragmented embryos.

Annexin V and TUNEL analysis

Eighty-three representative fragmented embryos were judged inappropriate for transfer or cryopreservation, and with patient permission were examined for indications of apoptosis. Eleven 2-cell embryos with type 2 fragmentation and which divided no further during an additional 24 h of culture (Figures 1B1 and 3T, asterisks) were examined. For 72 embryos, type 2 ($n = 34$, Figure 3N) or type 3 fragmentation ($n = 38$) occurred at the 4- to 8-cell stage (Figures 1E1 and 3U, asterisks). Living embryos were stained with propidium iodide ($n = 53$) to determine plasma membrane integrity and with annexin V ($n = 63$) to detect the movement of phosphatidylserine from the inner to the outer aspect of the plasma membrane, an early indicator of apoptosis. After annexin V analysis, fixed embryos were examined by TUNEL to detect DNA strand breaks and, if TUNEL-negative, were treated with DNase and reincubated in TUNEL reagents for use as positive controls. For these embryos, correspondence between TUNEL-positive fluorescence and DNA was demonstrated by DAPI staining. Figure 3N–S are representative images of a fragmented 8-cell embryo (Figure 3N, type 2 fragmentation observed at the 4-cell stage) stained with propidium iodide (Figure 3O), annexin V (Figure 3P) and examined by TUNEL (Figure 3Q). Propidium iodide-positive fragments, when present, were confined to a few small structures in the perivitelline space (Figure 3N and O, arrows) which, by light microscopy, appeared degenerate owing to the presence of an indistinct plasma membrane and translucent cytoplasm. Intense TUNEL-positive fluorescence was observed after treatment with DNase (Figure 3R, arrows) and the DNA specificity of the TUNEL signal was demonstrated by DAPI staining (Figure 3S, arrows). With the exception of an occasional, low-intensity patch of fluorescence, no annexin staining was associated with the numerous propidium-negative fragments, the residual blastomere from which the fragments developed, or with the blastomere(s) that appeared intact (Figure 1B2, D2 and E2). DNA strand break analysis performed

before immunostaining these embryos showed either no TUNEL signal (Figure 3U) or a single, intense focus of fluorescence which appeared to be the second polar body (Figure 3T; Figure 4A and C, arrows). A very similar pattern of fluorescence was observed for fragmented embryos that were first subjected to antibody analysis ($n = 48$) where, after immunostaining, TUNEL fluorescence was confined to a single polar body-sized fragment (Figure 4B and D). Perturbation of the polarized protein domains by the fragmentation process was confirmed in the same fragmented embryos that were analysed initially by annexin V and TUNEL staining and subsequently examined by scanning laser confocal immuno-fluorescence microscopy after incubation in appropriate antibodies (e.g. Figures 2R1, R2 and 3T; Figures 2S1–S3 and 3U). In the same respect, Figure 4A is representative of a relatively normal appearing embryo with two small clusters of fragments (type 4, Figure 4A, asterisks) which stained negatively for both annexin V and TUNEL (with the exception of the second polar body TUNEL fluorescence). After immuno-staining, the same embryo showed anti-Bax immunofluorescence in polarized domains in three of the four cells (Figure 2T1 and T2).

The occurrence of a positive TUNEL signal in some of the nuclei of 9/11 fragmented 8-cell embryos obtained from one single patient (Figure 4E) and 1/8 fragmented embryos from another was an exception to the absence of TUNEL fluorescence in the vast majority of fragmented embryos examined in this study. All 10 embryos exhibited type 2 fragmentation at the 2- to 4-cell stages, but continued to divide during the subsequent 48–60 h of culture. Between one and three nuclei per embryo in apparently normally sized blastomeres showed intense TUNEL fluorescence (Figure 4F, black asterisk). Here, the TUNEL-positive cells were occasionally difficult to image in the light microscope (Figure 4E, asterisk), but when identified were similar in appearance to blastomeres that were either severely damaged or lysed after thawing from a cryopreserved state. Typically, these cells did not appear fragmented or to be associated with fragments (Figure 4F, arrows) and stained positively with propidium iodide (data not shown), suggesting a loss of plasma membrane integrity. The diffuse fluorescence observed in this embryo is annexin V staining of membranous debris rather than discrete cytoplasmic fragments. The two TUNEL-positive structures indicated by white asterisks in Figure 4E and F are residual sperm heads.

Protein immunoblot (Western) analysis of Bax, Bcl-x and VEGF

While other investigators have described the occurrence of EGFR, c-kit and TGFβ2 protein in mammalian oocytes and early embryos (see above), before this report no similar information was available for Bax, Bcl-x and VEGF. An immunofluorescent examination of MII stage mouse oocytes for Bax (Figure 4W), Bcl-x (Figure 4X) and VEGF (Figure 4Y) provided preliminary evidence for the existence of these proteins and their polarized distributions in the mouse as well as human. Figure 4Z is a representative image of a MII mouse oocyte stained with non-specific mouse/rabbit primary and secondary antibodies. The results shown in this figure pertain
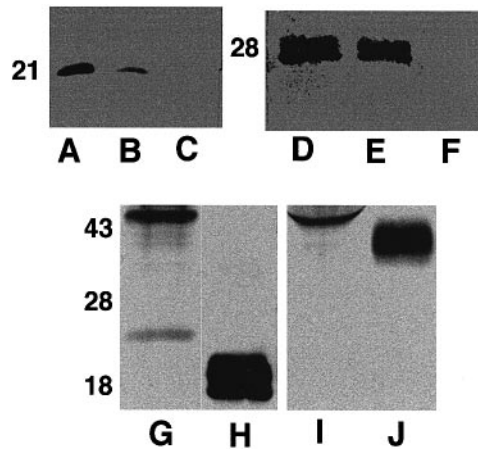
440

Figure 5. Lanes (**A**, **B**) Detection of an approximately 21 kDa protein following Western analysis of lysates prepared from 3T3-RSV control cells and murine GV stage oocytes, respectively, following immunodetection with an anti-Bax polyclonal antibody. Lanes (**D**, **E**) show the detection of approximately 28 kDa proteins following Western analysis of lysates prepared from 3T3-RSV control cells and murine GV stage oocytes, respectively, following immunodetection with an anti-Bcl-x polyclonal antibody. Lanes (**C**, **F**) Immunodetection of Bax- and Bcl-x-specific proteins, respectively, is abolished when primary antibodies are preincubated with blocking peptides before analysis of Western blots containing 3T3-RSV control cell lysates. Lane (**G**) Detection of approximately 22 and 44 kDa proteins; lane (**H**) detection of approximately 19 kDa proteins following Western analysis of murine GV stage oocyte lysates and VEGF protein standard, respectively, conducted under reducing conditions and immunodetected with an anti-VEGF monoclonal antibody. Lane (**I**) Detection of approximately 44 kDa proteins; lane (**J**) detection of approximately 38 kDa proteins following Western analysis of murine GV stage oocyte lysates and VEGF protein standards, respectively, conducted under non-reducing conditions and immunodetected with a second anti-VEGF monoclonal antibody.

to mouse primary antibody non-specific fluorescence, and comparable findings were obtained for non-specific rabbit primary antibodies (data not shown). To demonstrate the presence of specific proteins of expected size, cell lysates derived from GV stage mouse oocytes were examined by Western analysis for Bax, Bcl-x and VEGF with antibodies that are cross-reactive for both mouse and human. For these studies, typically, each analysis required between 600–800 fully denuded oocytes per lane, thus precluding any similar analyses using human materials. Western analysis of mouse oocyte lysates revealed a protein of approximately 21 kDa (Figure 5, lane A) when examined with a Bax-specific antibody; this protein co-migrated with a similarly sized band (lane B) in an adjacent lane containing a 3T3-RSV control cell lysate. Western analysis of oocyte lysates revealed a protein of approximately 28 kDa (lane D) when examined with a Bcl-x-specific antibody; this protein co-migrated with a similarly sized band (lane E) in a lane containing a 3T3-RSV control cell lysate. The antibody used in this analysis recognizes both the long (Bcl-x$_L$) and short form (Bcl-X$_S$) of the Bcl-x protein. In this study, only the long form of Bcl-x was detected by Western analysis. During both of these analyses, preincubation of primary antibodies with a 20-fold excess of blocking peptide eliminated the ability to detect Bax- and Bcl-x-specific bands

in 3T3-RSV control cell lysates (Figure 5, lanes C and F, respectively). The primary antibodies used for the detection of Bax and Bcl-x proteins during Western analysis were the same as those used during immunofluorescent analysis.

Immunoblots of oocyte lysates analysed under reducing conditions revealed bands of approximately 22 and 44 kDa (Figure 5, lane G) when examined with the same VEGF-specific monoclonal antibody used for immunofluorescent studies. These two bands likely represent the monomeric and dimeric forms of the VEGF protein in murine oocytes. Under reducing conditions, the 22 kDa VEGF-specific band detected in the oocyte lysates ran above two closely opposed bands of approximately 19 kDa detected in an adjacent lane containing an aliquot of an Sf 21-expressed VEGF$_{165}$ protein standard (lane H). Immunoblots of oocyte lysates analysed under non-reducing conditions revealed a band of approximately 44 kDa (lane I) when examined with a second VEGF-specific monoclonal antibody developed using Sf 21-expressed VEGF protein as the immunogen. Under non-reducing conditions, this second VEGF-specific antibody detected a band of approximately 38 kDa in an adjacent lane containing an aliquot of an Sf 21-expressed VEGF$_{165}$ protein standard (lane J). These results derived from analysis using two independent VEGF-specific monoclonal antibodies, and examination under reducing and non-reducing conditions confirm the presence of VEGF in oocytes and make possible the putative assignment of the VEGF isoform present in oocytes as VEGF$_{189}$. Further work is required to confirm this assignment.

Discussion

In clinical IVF, judgements of the 'quality' of human embryos are based primarily on several morphological criteria that can be assessed at the light microscopic level just before uterine transfer. Blastomere number, size and shape, and the presence or absence of extracellular fragments are the relevant characteristics that form the current basis for non-invasive evaluations of developmental competence (Giorgetti *et al.*, 1995). The degree of fragmentation is presently one of the most important empirical criteria used in human embryo assessments because the number and relative sizes of fragments can be readily estimated. Although some apparent differences in frequency have been reported, fragmentation has been observed to occur in the presence of different culture media and growth conditions, including the presence of a feeder layer (Van Blerkom, 1993, 1997; Wiemer *et al.*, 1993; Morgan *et al.*, 1995), suggesting that this phenomenon may be largely embryo-specific and not a function of a particular set of growth conditions. This interpretation is supported by reports of relatively high frequencies of fragmentation in human embryos where fertilization and development to day 5 occurred *in vivo* (Buster *et al.*, 1985).

With respect to developmental competence, the prevailing hypothesis is one that suggests that developmental viability declines as the number of fragments increases (Giorgetti *et al.*, 1995). Although a precise aetiology is unknown, fragmentation has been suggested to be a manifestation of developmentally lethal defects at the blastomere level associated with an

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instability within the cortical microfilament network (Van Blerkom, 1989), levels of ATP generation (Van Blerkom *et al.*, 1995b), chromosomal abnormalities including aneuploidy and mosaicism (Munne *et al.*, 1993) and apoptotic and necrotic processes (Juriscova *et al.*, 1996). In this regard, fragmentation may not have a common origin, but rather may be the overt manifestation of different underlying disorders. As noted here and by other investigators (e.g. Hoover *et al.*, 1995), normal births are known to have resulted from fragmented embryos in which numerous extracellular fragments were left undisturbed and therefore free to influence the fate of the affected embryos. This indicates that fragmentation *per se* is not an absolute determinant of developmental potential or lack thereof, with the possible exception of those instances where the level of fragmentation is so extensive that few, if any, blastomeres remain unaffected. As discussed below, our results suggest that the relationship between fragmentation and developmental potential may be associated with the specific pattern of fragmentation, the stage at which fragmentation occurs, and the particular blastomere(s) involved. This notion could explain why embryos with the same apparent degree of fragmentation can have very different developmental fates.

Different temporal and spatial aspects of blastomere fragmentation have differential effects on the complement of proteins located in polarized domains

Here we show that the phenomenon of regulatory protein polarization during early human development first observed for leptin and STAT3 also exists for the growth factors TGFβ2 and VEGF, the apoptosis-associated proteins Bcl-x and Bax, and the growth factor receptors c-kit and EGF-R. To the best of our knowledge, this is the first report demonstrating the presence of VEGF protein in human and mouse oocytes and early human embryos.

In this context, the term 'polarized' is meant to portray the asymmetric distribution of protein(s) in an oocyte, blastomere, or throughout the cells of an embryo. The characterization of proteins as residing in polarized domains is not meant to suggest the complete absence of a particular protein in a given location, but describes regions of relative abundance when addressing the overall distribution of a given protein during a particular stage of development. Indeed, it is important to note that while the most intense staining associated with each of the eight regulatory proteins examined in this study was associated with subplasmalemmal domains, intracellular staining for most of these proteins was observed using both fluorescent and non-fluorescent immunostaining methods. It should also be noted that the subplasmalemmal domains of the regulatory proteins examined in this study are quite large, involving roughly 50–70% of the cortex in the oocyte, as described previously for leptin and STAT3 (Antczak and Van Blerkom, 1997). The eight proteins investigated during this analysis were chosen because: (i) most have been shown to have important regulatory functions during early development in other systems, including the mammal (for review see Edwards and Beard, 1997); to date, the importance of STAT3 and EGF-R during early development has been demonstrated by peri-implantation embryonic demise in mutant mouse homo-

zygous for alleles containing targeted disruption of the respective gene (Threadgill *et al.*, 1995; Takeda *et al.*, 1997); (ii) they represent a diverse group of regulatory proteins; and (iii) with the exception of STAT3 and possibly leptin and VEGF (Cioffi *et al.*, 1997; Takeda *et al.*, 1997), transcripts for these proteins—or in some cases the proteins themselves—have been previously detected in the mammalian oocyte and early embryo, including Bax (Brenner *et al.*, 1997), EGF-R (Wiley *et al.*, 1992; Chia *et al.*, 1995), c-kit (Ismail *et al.*, 1997; Tanikawa *et al.*, 1998) and TGFβ2 (Schmid *et al.*, 1994; Ghiglieri *et al.*, 1995). However, the precise source(s) of the proteins in domains (pre-existing or newly translated) and their specific role(s) during early development have yet to be established.

The type and distribution of fragments within the embryo, the stage at which they occur, and the inclusion or exclusion of portions of regulatory protein domains in the fragments may provide important insights as to why particular fragmented human embryos have different developmental potentials. In the present study, fertilized eggs and cleavage stage human embryos were examined in detail and at high magnification on a video display by high-resolution light microscopy at approximately 8- to 12-h intervals after insemination. Embryos which showed varying degrees and patterns of fragmentation and which arrested cell division in culture, or which according to protocol were judged unsuitable for cryopreservation, were immunostained, analysed for apoptosis and—depending upon the type of secondary antibody conjugate—examined by light microscopy, standard epifluorescence microscopy or scanning laser confocal fluorescence microscopy. This multistage, multi-faceted examination made it possible to establish a chronology of events regarding the fragmentation of a portion of a blastomere and/or the fragmentation of an entire blastomere, and to a limited degree allowed us to relate certain aspects of fragmentation to developmental competence. Perhaps most importantly, this approach presented the opportunity to correlate fragmentation with the differential removal of peripherally located, polarized domains of regulatory proteins which we suggest may influence embryo developmental competence.

Detailed observations of cultured embryos demonstrated different temporal and spatial patterns of fragmentation that appear to have different consequences for the affected blastomere and for the developmental competence of the embryo as a whole. Pronuclear eggs in which a monolayer-like cluster of small fragments (type 1) formed in the hemisphere associated with the first and second polar bodies (animal pole) were virtually always capable of progressing through cleavage. It was not uncommon for a small cluster of type 1 fragments to originate at the 1-cell stage but to be clearly evident at the dissecting microscope level at 2-cell stage with no apparent increase in number or distribution detectable during subsequent cell divisions. No significant reduction in cell volume was associated with type 1 fragmentation and its occurrence appears to follow fertilization. To date, none of the several hundred MII oocytes denuded of coronal cells within 3 h of follicular aspiration, nor any of the over 500 unfertilized and unpenetrated oocytes examined within the first 24 h after insemination exhibited this or any of the other fragmentation patterns

described here. Less than 1% of unfertilized oocytes showed type 2 fragmentation, but this was observed after approximately 48–72 h of culture. Although the implantation potential of embryos with type 1 fragmentation could not be determined, blastocysts derived from these embryos appeared growth-retarded and defective with respect to inner cell mass organization and cell numbers (Van Blerkom, 1993). When fragmentation in this region of a pronuclear egg (polar body hemisphere) was extensive and involved the generation of columns of fragments (type 2), development beyond the 2-cell stage was rare, with most embryos remaining undivided. It is unknown whether these two patterns of fragmentation are extremes of the same mechanism, or represent different mechanisms by which the portions of the plasma membrane and subjacent cytoplasm are eliminated.

Immunofluorescent images demonstrated that polarized domains were significantly reduced (type 1) or, frequently, virtually eliminated (type 2) when fragmentation patterns originated from the hemisphere containing the polar bodies. A very similar perturbation of these domains occurred when type 1 fragmentation began at the 2-cell stage and involved the regions of both blastomeres in the vicinity of the second polar body. Although the implantation potential of these embryos could not be determined, most arrested their development during the cleavage stages. The complete detachment of some fragments from the plasma membrane was evident in serial optical sections, while others in the same region appeared to have some continuity with the ooplasm, suggesting that they were fixed during the process of formation. The persistence of what appeared to be the same fragments in an unchanged position during subsequent examinations suggests that it is unlikely that once eliminated from the cell, they can resorbed or in some fashion re-establish subplasmalemmal domains.

A third pattern of fragmentation (type 3) was observed primarily at or after the 4-cell stage. This pattern involved embryos which appeared normal at the 2- and 4-cell stages, but a blastomere(s) was then observed to fragment into a pleiomorphic population of cytoplasts when examined at the next cell division, i.e. at 4- and 6- to 8-cell stages, respectively. The developmental viability of embryos with type 3 fragmentation was demonstrated by births that occurred when all of the replaced embryos were of this type. The finding that fragmentation in such embryos is associated with different levels of diminished immunostaining for the proteins localized in subplasmalemmal domains may provide an important clue as to why some fragmented embryos of this type retain developmental viability while others do not.

A determination of the type and stage of fragmentation during regularly timed embryo inspections could have clinical applications in the selection of embryos for uterine transfer. For the proteins examined, the second polar body was virtually always associated with the domain-containing regions of the 1- and 2-cell embryo (Antczak and Van Blerkom, 1997), and remained associated with a particular blastomere through the preimplantation stages (Gardner, 1997). Therefore, this structure can serve as a useful reference or marker to orient the embryo with respect to these domains when types 1 and 2 fragmentation are observed and blastomeres remain largely intact. The possibility that fragmented embryos with higher developmental potential are those where fragmentation at the earliest stages involved the non-polarized domain portion of an egg or blastomere(s) is under investigation. However, it is important to emphasize that as these domains are currently defined, it is unknown whether other as yet unidentified proteins are localized to the non-domain regions of oocytes or the preimplantation embryonic cells observed in this or in our earlier study.

Our findings tend to suggest that the beneficial effects on outcome reported to occur after the mechanical removal of fragments from early cleavage stage embryos (Alikani *et al.*, 1993; Cohen *et al.*, 1994) may be more apparent than real. If the relationship between the distribution of immunofluorescent and non-immunofluorescent fragments described above is developmentally significant, then whatever effects fragmentation may have on embryo viability they are likely to have already occurred. Therefore, extraction of fragments would not be expected to alter developmental competence or repair whatever perturbation in cortical protein complement has ensued. The recent study of Dozortsev *et al.* (1998) on the impact of cellular fragmentation in early mouse embryos supports this conclusion because extraction of extracellular fragments provided no benefit for embryo development to the hatched blastocyst stage. It is possible that fragmentation, at some level, is a normal aspect of early human development but it is the extent or location, or both, of this phenomenon that influences developmental potential at the single cell and whole embryo levels.

Fragmentation and apoptosis

Several investigators have proposed that fragmentation in mouse oocytes (Fujino *et al.*, 1996; Perez and Tilly, 1997) or preimplantation human embryos (Juriscova *et al.*, 1996) is a manifestation of programmed cell death or apoptosis. Here, we suggest that fragmentation alone cannot be used as a definitive signal that an apoptotic process is involved. We present an alternative possibility, namely that apoptosis—if it is involved at all—may result from certain types of fragmentation, though the effects of fragmentation in this regard may not be immediately apparent in the developing embryo.

For approximately 20–25% of fragmented human embryos, Juriscova *et al.* (1996) observed intense foci of TUNEL fluorescence associated with extracellular fragments, and concluded that fragmentation was the result of an apoptotic process, involving the activation of a programmed cell death pathway. However, fragmented blastomeres classified as necrotic were also detected in this study, often within the same embryo in which another blastomere was characterized as apoptotic, and for approximately 75% of fragmented embryos examined, no TUNEL signal was detected. In the present report, fragmented cleavage stage embryos were examined with two markers of apoptosis: (i) annexin V, which is an early marker of apoptosis that detects phosphatidylserine residues translocated from the inner to the outer aspects of the plasma membrane (Martin *et al.*, 1995); and (ii) TUNEL, which detects 3′-OH DNA strand breaks that occur rapidly during apoptosis (Gavrieli *et al.*, 1991). Under the conditions

used during our analyses, virtually all intact and fragmented blastomeres, as well as the numerous extracellular fragments that were propidium iodide-negative, showed no TUNEL or annexin V fluorescence. After exposure to DNase, a coincident DAPI and TUNEL signal confirmed the specificity of this analysis, as shown previously for mouse and human oocytes (Van Blerkom and Davis, 1998). For most fragmented embryos, as well as for cleavage-arrested embryos that were retained in culture for several days after fragmentation was first observed (J.Van Blerkom and M.Antczak, unpublished results), detectable TUNEL fluorescence was largely confined to a single, small spheroidal body which, because of size and sole association with an intact blastomere, appeared to be the second polar body (Antczak and Van Blerkom, 1997; Gardner, 1997). An exception to this general finding was the detection of occasional TUNEL-positive nuclei in several fragmented embryos. Virtually all of these embryos were derived from a single patient and the TUNEL-positive cells were unusual insofar as they were of normal size and not surrounded by extracellular fragments. However, most of these cells, when examined closely in the light microscope, had an abnormally translucent cytoplasm that is consistent with a cell experiencing some form of autolysis rather than apoptosis. In support of this interpretation were the findings that these translucent cells stained positively with propidium iodide, and did not appear fragmented or to be closely associated with clearly detectable, membrane-bound fragments typical of the type 1–4 patterns. In this respect, it is important to note that a positive TUNEL signal can also occur in cells undergoing necrotic or autolytic cell death (Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp *et al.*, 1995). While we cannot exclude the possibility that these blastomeres are undergoing an atypical apoptotic process, it seems more likely that the positive TUNEL fluorescence observed is the result of DNA strand breaks caused by nuclease activation associated with autolysis or necrosis (Bicknell and Cohen, 1995).

At first glance, the light microscopic characteristics of some of the fragmented human embryos examined in this study do resemble some of the degenerative changes associated with normally occurring apoptotic processes (Tilly *et al.*, 1997). However, our results suggest that it is difficult to accept a global an assignment of apoptosis based solely on the presence of extracellular fragments (for review see Hockenberry, 1995) or a TUNEL-positive signal. In this regard, our findings with two different markers of apoptosis suggest that during the early stages of human development examined in this study, apoptosis and fragmentation do not appear to be concomitant processes. The apparent spatial, temporal and morphological differences in how blastomeres fragment suggest that different physiological processes may be involved. For example, the elaboration of numerous small spheroidal fragments of plasma membrane and subjacent cytoplasm from an affected blastomere which remains intact would appear to involve quite a different process from fragmentation characterized by the generation of columns of fragments or fragmentation resulting in the complete disruption of a cell. While the use of static images to decipher the nature of a dynamic process can be misleading, an interpretation of one of the findings of this study is that for some embryos, fragments can undergo successive rounds of re-fragmentation after their elimination from a blastomere. Both the generation of columns of fragments and the putative re-fragmentation of fragments are activities which have not been previously described or known to be associated with the formation of apoptotic bodies. In this respect, changes in cell physiology unrelated to apoptosis may cause some forms of fragmentation in the human embryo. Furthermore, the ability of blastomeres to continue to divide despite a reduction in cell volume associated with the elaboration of numerous small fragments (type 1 pattern) indicates that these cells are not undergoing apoptotic degeneration at the time of fragment formation.

If programmed cell death or apoptosis does indeed occur in human embryos, as has been suggested by others (Juriscova *et al.*, 1996), then perhaps the fragmentation process itself could initiate apoptosis in the affected blastomere. In this context, alterations of critical ratios of apoptosis-associated proteins such as Bax and Bcl-x (Oltvai *et al.*, 1993), for example, could result from the partial elimination of these proteins if portions of their domains were removed by fragmentation. While the consequence for such a blastomere might be apoptosis, for another fragmented blastomere(s) in the same embryo, one in which fragmentation did not involve these domains, or involved only a small portion, the effects of fragmentation might be negligible and the blastomere may remain unaffected, particularly if apoptotic proteins lost by fragmentation can to some extent be replaced by new synthesis from existing transcripts. Alternatively, cell degeneration by non-apoptotic processes may occur, perhaps as a result of significant cytoplasmic loss, such as that associated with type 2 fragmentation. A possibility currently under investigation is that some of the apoptotic events that occur during later developmental stages may result from forms of fragmentation that occurred shortly after fertilization or during early cleavage. For example, a type 1 pattern of fragmentation involving a pronuclear egg or one blastomere of a 2-cell embryo may affect particular protein domains so as to alter the potential and therefore the fate of successor cells, possibly resulting in their demise by way of an apoptotic pathway. Whether the underlying mechanism(s) responsible for the generation of spontaneous (Fujino *et al.*, 1996; Tilly *et al.*, 1997; Van Blerkom and Davis, 1998; Warner *et al.*, 1998) and experimentally induced forms of fragmentation (Perez *et al.*, 1997) observed in mouse oocytes is also responsible for the fragmentation phenotypes observed in human embryos remains to be determined. The aetiologies of fragmentation in mouse and human may involve different processes. In contrast to the mouse, newly aspirated human oocytes are rarely fragmented and spontaneous fragmentation of unfertilized and cultured oocytes rarely occurs (Van Blerkom and Davis, 1998).

Possible developmental influences of polarized proteins

We have previously shown that STAT3 (an activator of transcription) and leptin (a protein with diverse functions including fat metabolism, hypothalamic signalling, reproduction, haematopoiesis, angiogenesis and cognate cellular immune function: Bennett *et al.*, 1996; Chehab *et al.*, 1996;

Antczak *et al.*, 1997; Cioffi *et al.*, 1997; Lord *et al.*, 1998; Sierra-Honigmann *et al.*, 1998) occur in polarized domains in mouse and human oocytes and, after fertilization, become unequally distributed between the blastomeres of cleavage stage embryos (Antczak and Van Blerkom, 1997). During the first few cleavage divisions, a blastomere-specific pattern of leptin and STAT3 inheritance appears to be associated with the manner in which meridional and equatorial planes of cleavage (Edwards and Beard, 1997) interact with the polarized protein domains. For the blastocyst, the consequence of this unequal protein domain distribution during early development may subtend the differential distribution of leptin and STAT3 within the trophoblast and between the trophoblast and inner cell mass. In this previous report, we suggested that: (i) these two proteins may be derived from the cells of the cumulus oophorus and corona radiata rather than being the products of oocyte transcription; (ii) that the cell-specific pattern of leptin and STAT3 inheritance may have important roles in the progressive formation of the bilaminar preimplantation embryo; and (iii) that leptin and STAT3 may be representative of other regulatory proteins whose distribution in the oocyte and cells of the early embryo are also unequal and asymmetric. Here, we have examined the distribution of six additional regulatory proteins and found that representative growth factors, growth factor receptors and apoptosis-associated proteins are also localized to polarized cortical domains in the oocyte, and that the proteins within these domains are asymmetrically distributed to individual cells as development ensues. These findings suggest that the establishment of cortically located polarized protein domains in the oocyte may represent a fundamental mechanism designed to affect the course of early embryonic development. If this notion is correct, it is likely that this mechanism involves very broad and varied classes of proteins well beyond the scope of those examined here. In support of our findings on the EGF-R described for the human, Wiley *et al.* (1992) previously showed that the EGF-R displayed a polarized apical distribution in mature mouse oocytes and blastomeres of cleavage stage embryos. Careful inspection of the images presented in this 1992 study also indicates a differential distribution of EGF-R among blastomeres.

It is unknown when or how protein domains are established, or the amount of a protein that resides in a particular domain. Where transcripts for these proteins are known to exist in the early embryo, loss of a portion of the corresponding protein by fragmentation might not be expected to have adverse developmental effects, if the lost protein(s) can be appropriately restored. In contrast, depletion of classes of proteins which exist in domains but lack corresponding transcripts, represented by STAT3 and possibly leptin and VEGF, may have more severe consequences for embryonic development because these proteins cannot be replaced until embryonic transcription begins. The exact relationship between these transcripts and the domain forms of the proteins they encode is unknown. However, the results of this study clearly show that the localization of these polarized protein domains to the plasma membrane and subjacent cytoplasm makes them particularly susceptible to the membrane extrusions which occur during fragmentation. This is in contrast to the effects of fragmentation on proteins that are fairly evenly distributed throughout individual blastomeres. In these instances, as we have shown in the case of uvomorulin (E-cadherin), loss of affected protein(s) to the fragmentation process is less dramatic, and potentially less consequential. In this regard, the finding that the Bcl-x protein exists in a polarized domain was unexpected because this protein is currently thought to be localized almost exclusively to the outer mitochondrial membrane (Gonzalez-Garcia *et al.*, 1994). How Bax and Bcl-x are organized in polarized domains in oocytes and preimplantation embryos is under investigation and it is possible that some members of the Bcl-2 family, such as Bcl-x, may be involved in normal cellular processes unrelated to apoptosis (Rodger *et al.*, 1995).

The mechanism(s) involved and extent to which cell identity, fate and lineage are determined during early cleavage is unknown. Normal embryogenesis resulting from oocytes into which embryonic and adult nuclear transfers were performed (Stice and Keefer, 1993; Wilmut *et al.*, 1997; Wolf *et al.*, 1998) indicates either nuclear equivalence during cleavage or a plasticity inherent in blastomere and somatic cell nuclei when subjected to the unique influences of oocytes. Such influences may enable these nuclei to 'reprogramme' appropriately in synchrony with their new surroundings. Likewise, it is often assumed that blastomeres in early cleavage stage human embryos are transcriptionally equivalent, with translation driven primarily by oocyte-derived mRNA. In the human, significant transcription from the embryonic genome does not appear to begin until the 4- to 8-cell stages (Braude *et al.*, 1988). However, domains of important regulatory proteins may be progressively and unequally distributed to individual daughter cells beginning with the first cleavage division, as we have previously shown for leptin and STAT3.

If there is an interaction between these regulatory protein domains and the nuclei of individual blastomeres, then these domains may be a mechanism whereby nuclear equivalence is supplanted by correspondingly unique forms of transcriptional activation. Such activation could provide a basis for differential transcriptional activities, especially at the onset of embryonic gene expression (Palmieri *et al.*, 1994; Abdel-Rahman *et al.*, 1995), that influence cell identity, fate and lineage commitment as development ensues (Edwards and Beard, 1997). If this hypothesis is correct, then the occurrence of cell-specific differential gene expression may be governed by domains of proteins, some of which may be of maternal origin and prepositioned in the unfertilized oocyte. According to this interpretation, it is the manner in which these domains are partitioned to individual daughter cells during early cleavage that may generate blastomere-specific transcriptional activity, especially at the onset of embryonic gene expression. The study of fragmented human embryos may provide critical insights into both the potential roles of these regulatory proteins during early mammalian embryogenesis, and the extent to which the early development of mammals involves regulatory proteins and mechanisms similar to those described for other species.

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