Barcode tagging of human oocytes and embryos to prevent mix-ups in assisted reproduction technologies

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STUDY QUESTION: Is the attachment of biofunctionalized polysilicon barcodes to the outer surface of the zona pellucida an effective approach for the direct tagging and identification of human oocytes and embryos during assisted reproduction technologies (ARTs)?

SUMMARY ANSWER: The direct tagging system based on lectin-biofunctionalized polysilicon barcodes of micrometric dimensions is simple, safe and highly efficient, allowing the identification of human oocytes and embryos during the various procedures typically conducted during an assisted reproduction cycle.

WHAT IS KNOWN ALREADY: Measures to prevent mismatching errors (mix-ups) of the reproductive samples are currently in place in fertility clinics, but none of them are totally effective and several mix-up cases have been reported worldwide. Using a mouse model, our group has previously developed an effective direct embryo tagging system which does not interfere with the in vitro and in vivo development of the tagged embryos. This system has now been tested in human oocytes and embryos.

STUDY DESIGN, SIZE, DURATION: Fresh immature and mature fertilization-failed oocytes (n = 21) and cryopreserved day 1 embryos produced by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (n = 205) were donated by patients (n = 76) undergoing ARTs. In vitro development rates, embryo quality and post-vitrification survival were compared between tagged (n = 106) and non-tagged (control) embryos (n = 99). Barcode retention and identification rates were also calculated, both for embryos and for oocytes subjected to a simulated ICSI and parthenogenetic activation. Experiments were conducted from January 2012 to January 2013.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Barcodes were fabricated in polysilicon and biofunctionalized with wheat germ agglutinin lectin. Embryos were tagged with 10 barcodes and cultured in vitro until the blastocyst stage, when they were either differentially stained with propidium iodide and Hoechst or vitrified using the Cryotop method. Embryo quality was also analyzed by embryo grading and time-lapse monitoring. Injected oocytes were parthenogenetically activated using ionomycin and 6-dimethylaminopurine.

MAIN RESULTS AND THE ROLE OF CHANCE: Blastocyst development rates of tagged (27/58) and non-tagged embryos (24/51) were equivalent, and no significant differences in the timing of key morphokinetic parameters and the number of inner cell mass cells were detected between the two groups (tagged: 24.7 ± 2.5; non-tagged: 22.3 ± 1.9), indicating that preimplantation embryo potential and quality are not affected by the barcodes. Similarly, re-expansion rates of vitrified-warmed tagged (19/21) and non-tagged (16/19) blastocysts were similar. Global identification rates of 96.9 and 89.5% were obtained in fresh (mean barcode retention: 9.22 ± 0.13) and vitrified-warmed (mean barcode retention: 7.79 ± 0.35) tagged embryos, respectively, when simulating an automatic barcode reading process, though these rates were increased to 100% just by rotating the embryos during barcode reading. Only one of the oocytes lost one barcode during intracytoplasmic injection (100% identification rate) and all oocytes retained all the barcodes after parthenogenetic activation.
Introduction

Mismatching errors (mix-ups) of the reproductive samples in human assisted reproduction technologies (ARTs) have been reported in fertility clinics worldwide (Spriggs, 2003; Bender, 2006). Although sporadic, such incidents are devastating for those directly involved and a source of concern among future ARTs patients. This has led several authorities, scientific societies and fertility clinics to propose and implement measures to minimize the risk of mix-ups. In 2003, the Human Fertilisation and Embryology Authority (HFEA) introduced a mandatory manual double-witnessing protocol for all ART laboratory procedures in the UK (Human Fertilisation and Embryology Authority, 2003; Brison et al., 2004), a measure latter seconded by the European Society of Human Reproduction and Embryology (Magli et al., 2008). However, the effectiveness of the manual double-witnessing has been questioned because of the risk of involuntary automaticity (Toft and Mascie-Taylor, 2005). More recently, the HFEA proposed the use of electronic witnessing systems, which allow automation of the sample recognition process and identity verification (Adams and Carthey, 2006). These systems are based on the labeling of all labware used for each particular case with barcode stickers (MatcherTM, IMT, UK) or radio frequency identification labels (IVF WitnessTM, Research Instruments, UK), which can be identified by special readers connected to a computer. The risk of sample mismatching due to human error is minimized when using these systems, but as gametes and embryos are moved from one container to another several times during the course of an ART cycle, the possibility of misidentification still exists.

This gap in the control of sample traceability led us to propose a direct gamete/embryo tagging system in which the tag and the sample would move together throughout the whole ART process. As a first approach, we developed a direct embryo identification system based on the micro-injection of micro-sized polysilicon barcodes, which can be read under a standard inverted microscope, into the perivitelline space of mouse pronuclear embryos. This tagging method offered exciting results in terms of embryo viability and identification rates, but also limitations, such as the unexpected adhesion of the barcodes to the blastocyst cell surface after hatching (Novo et al., 2011). Subsequently, an alternative approach to overcome these limitations was conceived: the attachment of the barcodes to the outer surface of the zona pellucida (ZP) by means of their biofunctionalization with the wheat germ agglutinin (WGA) lectin (Penon et al., 2012; Novo et al., 2013a). Studies in mouse embryos have proved that this new tagging system is safe, as no detrimental effects in either in vitro or in vivo embryo development were observed, and highly efficient, as the barcodes could be successfully read in 100% of the tagged embryos (Novo et al., 2013a).

In the present work, the applicability of this newly developed tagging system to human oocytes and embryos donated for this particular research was tested. The validation of this technology was focused on two aims: to rule out potential detrimental effects of the barcode tags on embryo development, and to test the effectiveness of the tagging system during some of the laboratory procedures typically conducted in a human ART cycle. For the first purpose, the in vitro development of the tagged embryos was evaluated and compared with that of control non-tagged embryos in terms of development rates to the blastocyst stage, morphokinetic parameters assessed by time-lapse monitoring and embryo quality through morphological grading as well as blastocyst cell counting after differential staining. For the second purpose, retention of the barcodes attached to the zona pellucida and embryo identification rates were examined at several time-points during in vitro culture, after vitrification-warming procedures, and after a simulated oocyte intracytoplasmic sperm injection (ICSI).

Materials and Methods

Ethical approval

Experiments were conducted at the Universitat Autònoma de Barcelona and were approved by the Health Department of the Generalitat de Catalunya according to the Spanish Law governing Human Assisted Reproductive Technologies (14/2006).

Source of oocytes and embryos

Human immature oocytes and matured oocytes that failed to be fertilized after conventional in vitro fertilization (IVF) were used. These oocytes (n = 21), retrieved from five patients at the FecunMed-Granollers assisted reproduction center, were donated to research given their clinically useless nature. They were transported from the clinic to the lab (30 min trip) in G-MOPS medium (Vitrolife, Sweden) at room temperature and used immediately for tagging.

Cryopreserved (slow freezing) day 1 human embryos were donated for this particular research project by informed written consent signed by the patients. A total of 205 embryos, from 71 donors, were obtained from several assisted reproduction centers located close to the lab: FecunMed-Granollers, Dexeus, Teknon and GiroFIV. The embryos were produced, over several years, by either conventional IVF or ICSI, and were pooled...
together for the experiments. Embryos were thawed following the instructions of the Thaw-kit (Vitrolife, Sweden), transferred to a drop of G1 culture media (Vitrolife, Sweden) supplemented with human serum albumin (HSA; Vitrolife, Sweden) and placed in the incubator at 37 °C and 6% CO₂ until tagging.

Barcode fabrication and biofunctionalization

Barcodes are two-dimensional polysilicon microparticles with 10 μm in length, 6 μm in width and a thickness of 1 μm. They are asymmetric, to offer a start reading marker, and carry two rows of 4 rectangular bits of binary codification, which can be easily converted into a decimal number (Fig. 1a–c). The presence of 8 bits allows 256 different possible combinations (numbers 0–255). The fabrication of barcodes with more bits or with other shapes and dimensions could exponentially increase the number of possible combinations.

Barcodes were fabricated on 4″ p-type (100) silicon wafers through silicon microtechnologies used for microelectromechanical systems fabrication, as previously described in detail (Fernández-Rosas et al., 2009; Novo et al., 2011). They were biofunctionalized with the WGA lectin (Invitrogen, USA) to allow their attachment to the outer surface of the zona pellucida of oocytes and embryos (Fig. 1d–e). The biofunctionalization of polysilicon barcodes has also been thoroughly described (Penon et al., 2012; Novo et al., 2013a).

Oocyte and embryo tagging

Fresh oocytes and thawed embryos were tagged by the attachment of 10 WGA-biofunctionalized barcodes to the outer surface of their zona pellucida. First, a drop of PBS containing the barcodes was placed in a dish and groups of 10 barcodes were transferred, by micromanipulation, to separate drops of G-MOPS in the same dish. Next, the dish was placed under a stereoscopic microscope and one oocyte/embryo was transferred into each drop containing 10 barcodes. Each oocyte/embryo was rolled over the barcodes by means of an aspiration system, until the 10 barcodes were attached and uniformly distributed around its zona pellucida. The simple contact between the WGA-biofunctionalized barcodes and the zona pellucida outer surface was enough to allow their attachment, and it required only ~20 s per oocyte/embryo.

Oocyte intracytoplasmic injection and parthenogenetic activation

Tagged oocytes were microinjected without sperm to simulate an ICSI procedure. Sham ICSI was performed following the same steps as in a regular ICSI

![Figure 1](image-url) Design, dimensions and biofunctionalization of polysilicon barcodes. (a) Schematic representation of the barcodes used, showing shape, dimensions, number of bits, the start marker and the binary code number conversion into a decimal number. (b) Scanning electron microscope and (c) bright field images of a representative barcode (decimal number 105). Scale bar = 2 μm. (d) Schematic representation of the surface of a Wheat Germ Agglutinin (WGA)-biofunctionalized barcode and its interaction with the zona pellucida (ZP) glycoproteins. (e) Detail of a barcode attached to the outer surface of the zona pellucida of a human 1-cell embryo. A lower magnification image is shown in the inset. Scale bar = 10 μm.
Barcode tagging of human oocytes and embryos

Barcode tagging of human oocytes and embryos

procedure, except that no sperm was loaded into the ICSI pipette and just a small volume of G-MOPS medium was injected.

Tagged and injected mature oocytes were then parthenogenetically activated by sequential incubation in 5 μM ionomycin (Sigma, Spain) for 5 min and 2 mM 6-Dimethylaminopurine (Sigma, Spain) for 3 h in G1-HSA medium at 37°C and 6% CO2. Oocytes were cultured in G1-HSA at 37°C and 6% CO2 until 20 h post-ionomycin exposure, when they were evaluated for signs of activation (presence of pronuclei).

Embryo culture

Tagged and control (non-tagged) fertilized embryos were individually cultured in 20 μl microdrops of G1-HSA under oil (Vitrolife, Sweden) at 37°C and 6% CO2 from Day 1 to Day 3. On Day 3, embryos were washed with G-MOPS and transferred to G2 medium (Vitrolife, Sweden). Both media were supplemented with HSA. On Day 5, the medium was replaced with fresh G2-HSA medium.

Time-lapse monitoring

One-cell fertilized tagged and control embryos were subjected to automated time-lapse monitoring (PrimoVision, Vitrolife, Sweden) and digital images were taken every 10 min with intermittent white-light illumination. Embryos were cultured individually in the microwells of a specially designed well-of-the-well (WOW) dish (Vitrolife, Sweden) and were only manipulated for media changes on Day 3 and 5, when embryos were transferred to the same microwell position of another WOW dish containing the new medium.

Image sequences acquired for each embryo were analyzed using the PrimoVision Analyzer software (Vitrolife, Sweden) to determine the precise timing and duration of several developmental events. In particular, some of the kinetic parameters proposed by Kirkegaard et al. (2012) for time-lapse analysis of human embryos were evaluated. On the one hand, the timing of the disappearance of pronuclei, the first cleavage (2-cell stage time-point), the reappearance of nuclei after first cleavage, the second, third and fourth divisions (3-cell stage, 4-cell stage and 5-cell stage time-points, respectively), compaction, morula stage, blastocyst stage and full blastocyst stage were identified. On the other hand, the duration of the first cytokinesis, of the period between first cleavage and the reappearance of nuclei in the two blastomeres (reappearance of nuclei after first cleavage duration), and of the 2-cell and 3-cell stages were also annotated. Only the data for embryos reaching the full blastocyst stage were considered for calculating the time-points and durations of the developmental events analyzed. As the embryos used in our study came from both ICSI and conventional IVF procedures and the exact timing of their fertilization was unknown, thawing was considered as the start point and the time of all events is expressed as hours post-thawing.

Embryo morphological grading

Embryos were graded on Days 2, 3, 5 and 6 of their in vitro development according to the criteria for the morphological evaluation of human oocytes, early embryos and blastocysts, proposed by the Asociación para el Estudio de la Biología de la Reproducción (2008). The evaluation was performed on acquired images and embryos were categorized in four grades, from A (embryo with optimal quality and the best implantation potential) to D (a poor-quality embryo with a low chance of implantation).

Day 2 and Day 3 embryos were assigned to a particular category depending on the number of cells, symmetry and granularity of the blastomeres, percentage of fragmentation, presence of multinucleated blastomeres and presence of vacuoles. Blastocysts were categorized based on the day of formation (Day S: categories A and B; Day 6: categories C and D) and the inner cell mass (ICM), trophoderm (TE) and blastocoel morphology.

Blastocyst evaluation by differential staining

The cellular composition of Day 6 blastocysts was assessed by differential staining of ICM and TE cells using a modification of the protocol described by Thouas et al. (2001). Briefly, blastocysts were first incubated in PBS with 1% (v/v) Triton X-100 and 100 μg/ml propidium iodide (Sigma, Spain) for up to 20 s. Then, they were immediately transferred into a fixative solution of 100% ethanol containing 25 μg/ml 33,258 Hoechst (Sigma, Spain) and stored in this solution at 4°C overnight. Fixed and stained blastocysts were mounted on a slide in a 3 μl drop of Vectashield (Vectorlabs, USA) and then flattened with a coverslip. Cell counting was performed manually using the z-projected images obtained from xyz hyperstack scan by an SP5 confocal laser-scanning microscope (Leica, Germany). The composite image resulting from the capture of the two channels showed ICM and TE nuclei as blue and red/pink, respectively.

Embryo vitrification and warming

Expanded blastocysts were vitrified and warmed according to protocols previously described by Kuwayama et al. (2005), using the Cryotop device and commercially available vitrification and thawing solutions (Kitazato Bio-Pharma Co., Japan). After warming, blastocysts were transferred to droplets of G2-HSA under mineral oil and cultured at 37°C and 6% CO2 for up to 24 h.

Statistical analysis

All experiments were repeated at least three times on separate days and the results were pooled. In vitro development rates and blastocysts post-warming re-expansion rates were analyzed by the Fisher’s exact test. Identification rates were analyzed by ANOVA. The values of barcode retention were compared using Kruskal–Wallis and Dunn’s tests. Embryo quality grading results were analyzed by Chi-squared test for independence (Days 2 and 3) and Fisher’s exact test (Days 5 and 6). Finally, cell counts after blastocyst differential staining and morphokinetic parameters were compared between tagged and control embryos using either Mann–Whitney or Student’s t-test, as appropriate. A P-value < 0.05 was considered statistically significant.

Results

Tagging system evaluation during in vitro embryo culture

To test the validity and safety of the tagging system in human embryos, 58 pronuclear stage embryos were tagged, in five experimental replicates, by the attachment of 10 WGA-biofunctionalized barcodes to the outer surface of their zona pellucida. Then, they were cultured in vitro for 6 days (120 h) in separate microdrops, to monitor each embryo individually (Fig. 2), in parallel with a group of control non-tagged embryos (n = 51). The development rates of tagged and control embryos were equivalent at all stages, and a similar blastocyst rate was achieved in the two groups (46.7%, 27/58 tagged embryos; 47.1%, 24/51 control embryos).

The number of barcodes remaining attached to the zona pellucida of the developed tagged embryos (mean retention) was recorded every 24 h (Table I). Only three embryos (5.2%) lost one of the barcodes while being transferred from the drops where tagging was performed to the drops of culture medium (0 h, Day 1 of culture). No significant losses were detected after media change on Day 3 (48–72 h) or on Day 5 (96–120 h). However, a small number of barcodes were progressively lost during embryo culture and after 72 h of culture (Day 4) the mean retention value, despite being high (9.54 ± 0.12 barcodes per...
Figure 2. In vitro development of tagged embryos and identification process. Two different focal planes of the same embryo observed on Day 1 (a1 and a2), Day 2 (b1 and b2), Day 3 (c1 and c2), Day 4 (d1 and d2), Day 5 (e1 and e2) and Day 6 (f1 and f2), with barcodes attached to its zona pellucida, are shown. Identification was performed simply by adjusting the focus of the inverted microscope (× 200 magnification) until a barcode properly oriented for reading was found (white circles). A magnified image of the readable barcode (binary code = 0000 0000; decimal number = 0) is shown in the insets. Scale bar = 20 μm.

Table I. Barcode retention after manipulation and during in vitro culture of tagged embryos.

<table>
<thead>
<tr>
<th>Number of barcodes per embryo</th>
<th>Number of tagged embryos</th>
<th>Number of developed embryos with barcodes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h (Day 1)</td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>55 (94.8)</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>3 (5.2)</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>≤6</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

Mean retention ± SEM^a

| 10^b | 9.95 ± 0.03^b | 9.81 ± 0.07^b,c | 9.68 ± 0.09^b,c | 9.54 ± 0.12^c,d | 9.18 ± 0.14^d | 9.22 ± 0.13^d |

SEM, standard error of the mean.

^aMean number of barcodes attached per embryo.

^b,c,dDifferent superscripts denote significant differences among mean retention values at different time-points (P < 0.05; Kruskal–Wallis, Dunn’s post hoc test).
Barcode tagging of human oocytes and embryos

Barcode could be read under the inverted microscope (200× magnification; identification rate) was also recorded every 24 h. It is important to note that, to mimic an eventual automatic barcode reading process, the dish containing the embryos was placed under an inverted microscope and the reading of the barcodes was performed only by adjusting the focus, without any embryo manipulation. Hence, only embryos with at least one barcode in a correct spatial orientation for reading could be successfully identified (Fig. 2).

Total identification rates were high and similar at all time points analyzed (90.9–100%; Table II). As expected, they decreased as the number of barcodes attached per embryo decreased, although significant differences were only observed in embryos with eight or less barcodes. Regardless of the number of barcodes attached per embryo and the culture time-point, embryo identification was successful in 96.9% of the total analyses realized (n = 256) under the conditions used in our study. However, it is important to point out that 100% of the embryos could be successfully identified simply by rotating them to allow the correct spatial orientation of at least one of their barcodes.

Embryo quality was also assessed in this first set of experiments, using two different quality assays: embryo grading after morphological evaluation on Days 2, 3, 5 and 6 (Table III, experiment 1) and Day 6 blastocyst cell counts after differential staining (Table IV). No differences were observed between tagged and control embryos in either of the two quality assays applied.

Morphokinetic evaluation of tagged embryos

In a second set of experiments (six replicates), 1-cell tagged (n = 48) and control non-tagged (n = 48) embryos were cultured for up to 6 days in separate microwells and subjected to automated time-lapse monitoring for their morphokinetic evaluation. In these experiments, barcode retention and identification rates of tagged embryos were exclusively evaluated at the expanded blastocyst stage, once the embryo morphokinetic evaluation was completed.

No differences were found between the two groups of embryos for any of the morphokinetic parameters analyzed (Table VI). At the end of the culture, 43.8% (21/48) of the tagged embryos achieved the blastocyst stage, a value equivalent to the 39.6% (19/48) of the control group and to the 46.7% obtained in the microdrop-cultured tagged embryos of the first set of experiments. In the tagged blastocysts, the barcode mean retention value was 9.50 ± 0.16 and the identification rate was 90.9%, both values again equivalent to those obtained in the first set of experiments using microdrop cultures (120 h, Day 6; Tables I and II).

As in the first set of experiments, embryo morphology was evaluated, graded and compared between tagged and control embryos on Days 2, 3, 5 and 6, and no differences were observed between the two groups (Table III, experiment 2).

Tagging system evaluation after vitrification/warming procedures

To test the validity of the tagging system after a vitrification process, tagged (n = 21) and control (n = 19) Day 5 and 6 expanded blastocysts produced in the second set of experiments were vitrified once their morphokinetic evaluation was completed. The number of barcodes attached to each tagged blastocyst was recorded before vitrification, and a mean number of 9.48 ± 0.16 barcodes/blastocyst was observed (Table VI). After warming, blastocysts were analyzed at 3 h and 24 h to determine the number of re-expanded blastocysts, which was equivalent between tagged and control blastocysts. Moreover, both for control and for tagged embryos, re-expansion rates were equivalent between Day 5 and Day 6 vitrified blastocysts at both time-points analyzed (Table VI).

Barcode retention mean values and embryo identification rates were also calculated at both time-points after warming. Vitrification and warming procedures led to a significant decrease in the mean number of barcodes attached per embryo (Table VI). However, after warming, no more significant barcode losses occurred during culture, and at least 7 (range: 7–10) and 5 (range: 5–9) barcodes per embryo remained

<p>| Table II Identification rates of tagged embryos at different time-points during in vitro culture. |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Number of barcodes | Number of developed embryos successfully identified (%) | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th>per embryo</th>
<th>0 h (Day 1)</th>
<th>24 h (Day 2)</th>
<th>48 h (Day 3)</th>
<th>72 h (Day 4)</th>
<th>96 h (Day 5)</th>
<th>120 h (Day 6)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>55/55 (100)</td>
<td>44/45 (97.8)</td>
<td>35/35 (100)</td>
<td>25/25 (100)</td>
<td>13/13 (100)</td>
<td>10/10 (100)</td>
<td>182/183 (99.5)a</td>
</tr>
<tr>
<td>9</td>
<td>2/3 (66.7)</td>
<td>6/7 (100)</td>
<td>10/10 (100)</td>
<td>11/11 (100)</td>
<td>13/14 (92.9)</td>
<td>13/13 (100)</td>
<td>55/57 (96.5)ab</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>1/1 (100)</td>
<td>2/2 (100)</td>
<td>3/5 (60.0)</td>
<td>2/4 (50.0)</td>
<td>8/12 (66.7)b</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
<td>1/1 (100)</td>
<td>1/1 (100)</td>
<td>–</td>
<td>3/4 (75.0)</td>
</tr>
<tr>
<td>Total</td>
<td>57/58 (98.3)</td>
<td>51/53 (96.2)</td>
<td>46/47 (97.9)</td>
<td>39/39 (100)</td>
<td>30/33 (90.9)</td>
<td>25/27 (92.6)</td>
<td>248/256 (96.9)</td>
</tr>
</tbody>
</table>

No significant differences among total identification rates at different time-points were detected.

abValues with different superscripts significantly differ within the same column (P < 0.05; ANOVA).
attached after 24 h of culture in Day 5 and Day 6 warmed blastocysts, respectively. At the end of the culture, the mean barcode retention value in vitrified-warmed blastocysts ($7.79 + 0.35$; Table VI) was significantly lower than that obtained in the first set of experiments in non-vitrified blastocysts ($9.22 + 0.13$; Table I).

As observed in non-vitrified embryos, the identification rate of vitrified-warmed embryos was directly related to the number of barcodes that remained attached to their zona pellucida, decreasing as the number of barcodes decreased ($83.3–91.7$%; Table III). However, because the integrity of all the barcodes was preserved during the vitrification-warming procedures and all the embryos had at least three barcodes attached after warming, an identification rate of 100% could be achieved simply by rotating the non-identifiable embryos until one of the remaining barcodes was properly oriented for reading.

### Tagging system evaluation after oocyte intracytoplasmic injection and parthenogenetic activation

Immature and mature oocytes ($n = 21$) tagged with $10$ barcodes were microinjected without sperm to simulate an ICSI procedure (Fig. 3) and the number of barcodes that remained attached after microinjection was recorded to evaluate the possible effects of this manipulation on barcode retention. Only one of the oocytes lost one barcode during the micromanipulation process, so the mean barcode retention was $9.95 + 0.05$ barcodes per oocyte, with an identification rate of $100$%.

Then, mature oocytes were selected ($n = 13$) and parthenogenetically activated. The number of barcodes remaining attached to the zona pellucida of the activated oocytes (20 h post-ionomycin exposure) was again recorded, as an indicator of the possible effects on barcode retention of the zona pellucida modifications that take place during the zona reaction. Nine of the mature oocytes were successfully activated, as judged by the presence of pronuclei 20 h later, and all of them retained all the barcodes during the activation process.

### Discussion

In the present work, the applicability in human oocytes and embryos of a direct tagging system previously developed by our group in mouse embryos (Novo et al., 2013a) was tested. First, we evaluated the safety of the tagging system by assessing potential effects on the in vitro
Barcode tagging of human oocytes and embryos

Table V  Morphokinetic parameters of control and tagged embryos developed up to full blastocyst stage.

<table>
<thead>
<tr>
<th>Parameter analyzed</th>
<th>Time (h ± SEM (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disappearance of pronuclei time-point</td>
<td>Control 9.1 ± 0.6 (16)  Tagged 8.2 ± 0.7 (19)</td>
</tr>
<tr>
<td>1st cytokinesis duration</td>
<td>0.45 ± 0.03 (19)  0.47 ± 0.05 (21)</td>
</tr>
<tr>
<td>2-Cell stage time-point</td>
<td>13.9 ± 1.8 (19)   11.9 ± 0.8 (21)</td>
</tr>
<tr>
<td>Reappearance of nuclei after first cleavage time-point</td>
<td>15.2 ± 0.9 (10)   16.1 ± 1.5 (13)</td>
</tr>
<tr>
<td>Reappearance of nuclei after first cleavage duration</td>
<td>3.0 ± 0.4 (10)   3.3 ± 0.5 (13)</td>
</tr>
<tr>
<td>3-Cell stage time-point</td>
<td>26.5 ± 2.8 (17)   24.6 ± 1.3 (18)</td>
</tr>
<tr>
<td>2-Cell stage duration</td>
<td>12.6 ± 0.6 (17)   12.7 ± 0.7 (18)</td>
</tr>
<tr>
<td>4-Cell stage time-point</td>
<td>27.4 ± 2.4 (19)   26.2 ± 1.2 (21)</td>
</tr>
<tr>
<td>3-Cell stage duration</td>
<td>1.0 ± 0.2 (17)    1.3 ± 0.3 (18)</td>
</tr>
<tr>
<td>5-Cell stage time-point</td>
<td>39.2 ± 2.6 (19)   41.8 ± 2.2 (21)</td>
</tr>
<tr>
<td>Compaction time-point</td>
<td>77.2 ± 1.9 (19)   75.2 ± 1.4 (21)</td>
</tr>
<tr>
<td>Morula time-point</td>
<td>85.4 ± 2.0 (19)   84.0 ± 1.7 (21)</td>
</tr>
<tr>
<td>Blastocyst time-point</td>
<td>94.8 ± 2.0 (19)   92.0 ± 1.9 (21)</td>
</tr>
<tr>
<td>Full Blastocyst time-point</td>
<td>104.1 ± 2.4 (19)  101.2 ± 2.3 (21)</td>
</tr>
</tbody>
</table>

SEM, standard error of the mean. No significant differences were detected for any parameter analyzed between control and tagged embryos (P > 0.05; Mann–Whitney and Student’s t-test).

Table VI  Post-warming re-expansion rates, barcode mean retention values and identification rates of vitrified tagged blastocyst.

| Group          | 3 h post-warming | 24 h post-warming | ID (%) | Mean retention 
<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>19</td>
<td>11</td>
<td>17 (81.0)</td>
</tr>
<tr>
<td>Tagged</td>
<td>12</td>
<td>20</td>
<td>12</td>
<td>19 (95.2)</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>39</td>
<td>23</td>
<td>36 (94.8)</td>
</tr>
</tbody>
</table>

No significant differences in the identification rates of tagged blastocysts were detected among the different time-points (P > 0.05, ANOVA). No significant difference in the re-expansion rates were detected between the control and the tagged embryos.

Development and quality of human embryos. The development rates of tagged embryos were equivalent at all stages to those of control embryos. Similarly, the quality of the tagged embryos, as judged by their morphological grading at several developmental stages and by the cell counts in Day 6 blastocysts, was equivalent to that of control embryos. For a more exhaustive and precise analysis of embryo competence, a morphokinetic evaluation of tagged embryos by time-lapse monitoring was also performed. This evaluation revealed no alterations in the timing and duration of several developmental events in the tagged embryos that achieved the blastocyst stage, when compared with control embryos, corroborating the results of our other tests of embryo developmental potential and quality. Therefore, according to all the analyses performed, tagging of human embryos with WGA-biofunctionalized barcodes does not have any significant effect on embryo preimplantation developmental and quality, in agreement with our previous results in mouse and bovine embryos (Novo et al., 2013a,b). Potential effects of the WGA-biofunctionalized barcodes on implantation and on post-implantation development of human tagged embryos were not analyzed in the present study and cannot be ruled out unless embryo transfers are performed. However, previous studies in mouse embryos have clearly demonstrated the absence of detrimental effects in full-term development (Novo et al., 2013a). In parallel to the safety of the system, we also investigated its effectiveness for the labeling and identification of human oocytes and embryos. For this purpose, some of the key laboratory procedures typically conducted during the course of an ART cycle were carried out with tagged oocytes and embryos, and the retention of the barcodes on the
zona pellucida as well as the identification rates of the tagged samples at several points of the procedures were determined. A small, though significant, progressive loss of barcodes occurred during embryo culture, as we had previously observed in mouse embryos (Novo et al., 2013a). This loss was not caused by embryo manipulations during sequential culture, and could probably be attributed to a slight weakening of the WGA-ZP binding resulting from the modifications that normally occur in the zona pellucida during early embryo development (Vanroose et al., 2000). Despite these losses, barcode mean retention values at the end of the culture were high (9.22 ± 0.13 barcodes/embryo), and equivalent to those previously observed in mouse tagged embryos (9.56 ± 0.06 barcodes/embryo) (Novo et al., 2013a). High retention values of WGA-biofunctionalized barcodes were indeed expected, as a high affinity of WGA to the zona pellucida of both mouse (Skutelsky et al., 1994; Novo et al., 2013c) and human (Maymon et al., 1994; Talevi et al., 1997; Jiménez-Movilla et al., 2004) oocytes and embryos has been documented. It is worth noting, however, that once the embryos hatched from the zona pellucida all of them were freed from barcodes, which remained attached to the empty zona pellucida.

Retention of at least seven barcodes per embryo during culture facilitated the achievement of a very high global identification rate (96.9%), which was also similar to the rate obtained in our earlier study in mouse embryos (96.5%) (Novo et al., 2013a). However, as previously pointed out, the simple rotation of the non-identified embryos until one of their barcodes became properly oriented for reading could have led to a 100% identification rate. We chose not to manipulate the embryos during the identification process because, although manual eye reading of the barcodes was performed in the present study, we wanted to simulate an eventual process in which the barcode would be automatically read by a computer software just by placing the embryo under the inverted microscope and focusing on one of its attached barcodes. This software has already been developed and is currently being tested in our laboratory. Irrespective of the barcode reading system (manual or automatic), an alternative to embryo rotation during the reading process in those few embryos that cannot be directly identified would be to increase the number of barcodes initially attached per embryo (>10). This would increase the probability that, regardless of embryo orientation, at least one of the barcodes attached to its zona pellucida remains properly oriented for reading.

The effectiveness of the embryo tagging system under another commonly performed procedure during the course of an ART cycle, embryo cryopreservation, was also investigated. As expected, the viability of the vitrified-warmed blastocysts was not affected by the presence of the barcode tags, similar to what we had previously observed in slow-frozen tagged mouse embryos (Novo et al., 2013a). However, a significant decrease in the barcode mean retention values was observed after warming, possibly associated with the physical damage (Van den Abbeel and Van Steirteghem, 2000) and the biochemical modifications (Bogliolo et al., 2012) that the zona pellucida undergoes during cryopreservation procedures, both of which may affect the WGA-ZP binding interactions. In fact, a mean of 1.5 barcodes per embryo detached after vitrification-warming of human embryos, which was equivalent to the mean number of barcodes detached after the slow freezing-thawing of mouse embryos (Novo et al., 2013a).

As identification rates are directly related to the number of barcodes present in the zona pellucida when embryos are not manipulated during the barcode reading process, a decrease in the barcode mean retention values after vitrification-warming was paralleled by a decrease in embryo identification rates when compared with non-cryopreserved blastocysts, though this decrease was not significant. Therefore, the effectiveness of the tagging system is slightly reduced after embryo cryopreservation but, again, it could be increased to 100% simply by rotating the embryos.

**Figure 3** Intracytoplasmic sham injection of tagged oocytes. (a–d) Image sequence of a tagged oocyte during microinjection. (e and f) Two different focal planes of the oocyte after injection, with the 10 barcodes still attached to its zona pellucida. Scale bar = 20 μm.
Barcode tagging of human oocytes and embryos

Javier Nadal (Centro Médico Teknon) and Joan Sarquella (GiroFIV) for providing the oocytes and embryos used in this study.

Authors’ roles

J.A.P. and J.E. designed the barcodes. L.P.-G. and A.E. designed the biofunctionalization of the barcodes. S.N., C.N., L.B., J.S. and E.I. designed the biological experiments. R.G.-M. fabricated the barcodes. O.P. biofunctionalized the barcodes. S.N. carried out the experiments with human oocytes and embryos. C.N., L.B. and E.I. supervised the experiments. S.N. and L.B. performed the statistical analyses. S.N. and E.I. wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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Conflict of interest

None declared.

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