Direct embryo tagging and identification system by attachment of biofunctionalized polysilicon barcodes to the zona pellucida of mouse embryos

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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 cultured embryos?

SUMMARY ANSWER: The results achieved provide a proof of concept for a direct embryo tagging system using biofunctionalized polysilicon barcodes, which could help to minimize the risk of mismatching errors (mix-ups) in human assisted reproduction technologies.

WHAT IS KNOWN ALREADY: Even though the occurrence of mix-ups is rare, several cases have been reported in fertility clinics around the world. Measures to prevent the risk of mix-ups in human assisted reproduction technologies are therefore required.

STUDY DESIGN, SIZE, DURATION: Mouse embryos were tagged with 10 barcodes and the effectiveness of the tagging system was tested during fresh in vitro culture (n=140) and after embryo cryopreservation (n=84). Finally, the full-term development of tagged embryos was evaluated (n=105).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Mouse pronuclear embryos were individually rolled over wheat germ agglutinin-biofunctionalized polysilicon barcodes to distribute them uniformly around the ZONA PELLUCIDA surface. Embryo viability and retention of barcodes were determined during 96 h of culture. The identification of tagged embryos was performed every 24 h in an inverted microscope and without embryo manipulation to simulate an automatic reading procedure. Full-term development of the tagged embryos was assessed after their transfer to pseudo-pregnant females. To test the validity of the embryo tagging system after a cryopreservation process, tagged embryos were frozen at the 2-cell stage using a slow freezing protocol, and followed in culture for 72 h after thawing.

MAIN RESULTS AND THE ROLE OF CHANCE: Neither the in vitro or in vivo development of tagged embryos was adversely affected. The tagging system also proved effective during an embryo cryopreservation process. Global identification rates higher than 96 and 92% in fresh and frozen-thawed tagged embryos, respectively, were obtained when simulating an automatic barcode reading system, although these rates could be increased to 100% by simply rotating the embryos during the reading process.

LIMITATIONS, REASONS FOR CAUTION: The direct embryo tagging developed here has exclusively been tested in mouse embryos. Its effectiveness in other species, such as the human, is currently being tested.
WIDER IMPLICATIONS OF THE FINDINGS: The direct embryo tagging system developed here, once tested in human embryos, could provide fertility clinics with a novel tool to reduce the risk of mix-ups in human assisted reproduction technologies.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by Spanish Ministry of Education and Science (TEC2011-29140-C03) and by the Generalitat de Catalunya (2009SGR-00282). The authors do not have any competing interest.

Key words: assisted reproductive technologies (ART) / embryo tagging / mix-ups / traceability / biofunctionalization

Introduction

The unequivocal identification of reproductive samples is fundamental to minimize the risk of mismatching errors (mix-ups) in human assisted reproduction technologies (ARTs). Even though the occurrence of mix-ups is rare, several cases have been reported in fertility clinics around the world (Spriggs, 2003; Bender, 2006). Currently applied measures, such as the labeling of all labware together with the implementation of manual double witnessing (Brison et al., 2004; Magli et al., 2008) or electronic witnessing protocols (Schnaufer et al., 2005; Glew et al., 2006), undoubtedly minimize the risk of sample mismatching due to human error. However, as gametes/embryos are moved from one container to another several times during the course of an ART cycle, the possibility of misidentification still exists. Furthermore, the implementation of these measures increases the already high workload of embryologists and clinicians and the costs of ART procedures.

These limitations led us to propose a direct gamete/embryo tagging method in which the tag and the biological sample would move together throughout the entire ART process, from oocyte collection to embryo transfer back to the patient. As a first approach, our group developed a direct embryo identification system based on the microinjection of micrometric polysilicon barcodes, which can be read under a standard inverted microscope, into the perivitelline space of mouse pronuclear embryos (Novo et al., 2011). Despite of the good results achieved with this system in terms of embryo viability and identification rates, it was observed that some barcodes remained adhered to the cells surface after embryo hatching. Although the effects of the adhered barcodes on embryo implantation were not assessed, this situation should be avoided if such an embryo tagging system will eventually be applied in a clinical setting. Consequently, to overcome this limitation, a new approach has been devised to avoid the direct contact of the barcodes with the embryo cells: the attachment of the polysilicon barcodes to the outer surface of the zona pellucida (ZP).

The ZP is an extracellular glycoprotein coat that surrounds mammalian oocytes and preimplantation embryos up to the hatching stage, which precedes embryo implantation into the wall of the maternal uterus (Wassarman, 2008). Therefore, as the ZP is present for the whole period during which oocytes/embryos are maintained in the laboratory when performing ARTs, it appears to be an ideal structure to associate the barcodes with, as this would avoid the direct contact of the barcodes with the embryo cell surface. In this sense, the biochemical modification of the polysilicon surface by means of a functionalization is needed to achieve the attachment of the barcodes to the outer surface of the ZP. In previous studies performed in our laboratory several biomolecules with ZP-binding capacity, namely an anti-ZP2 antibody and the lectin wheat germ agglutinin (WGA) and phytohemagglutinin-L, were tested as possible candidates for barcode biofunctionalization. WGA proved to be the most appropriate biomolecule, among the three candidates tested, to ensure a robust and stable attachment of microparticles to the ZP of mouse oocytes and embryos, through zona reaction and in vitro culture up to the blastocyst stage (unpublished results). WGA shows its highest affinity to N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid monosaccharides (Bhavanandan and Katic, 1979), abundant in the ZP of most mammalian species (Skutelsky et al., 1994; Habermann et al., 2011). Next, the biofunctionalization of the polysilicon barcodes with the WGA lectin was optimized, and we found that barcodes with high surface roughness and covalently biofunctionalized using triethoxysilylundecanal (TESUD) as the linker offered excellent attachment and retention rates onto the mouse embryo ZP (Penon et al., 2012).

The present work constitutes the last step in the development of the new embryo tagging system based on the attachment of WGA-biofunctionalized barcodes to the outer surface of the ZP, and its aim is the validation of this system in mouse embryos. With this purpose, WGA-biofunctionalized barcodes were attached to the ZP of mouse pronuclear embryos and the following parameters were evaluated: (i) the in vitro and full-term developmental potential of tagged embryos; (ii) the in vitro developmental potential of cryopreserved tagged embryos; (iii) the stability of barcode attachment to the ZP during in vitro culture of fresh and cryopreserved tagged embryos; (iv) the rate of embryo identification by means of barcode reading under an inverted microscope and (v) the release of the barcodes after blastocyst hatching.

Materials and Methods

The animal care and procedures employed in this study were performed according to protocols approved by the Departament d’Agricultura, Ramadera i Pesca de la Generalitat de Catalunya and by the Ethics Committee on Animal and Human Research of the Universitat Autònoma de Barcelona.

Barcode design and fabrication

Barcodes are two-dimensional polysilicon microparticles with 10 μm in length and 6 μm in width and with a thickness of 0.5 μm. They are asymmetric to offer a start reading marker, and carry eight rectangular bits of binary codification, which allows 256 different possible combinations (numbers 0 to 255). The binary codification of the barcodes can be easily converted into a decimal number (Fig. 1). These barcodes have already been used by our group to tag mouse embryos by their microinjection into the perivitelline space (Novo et al., 2011).

Barcodes were fabricated on 4″ p-type (100) silicon wafers using silicon microtechnologies used for microelectromechanical system fabrication.

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Barcode biofunctionalization

All the reactions were carried out using a suspension of barcodes in the appropriate solutions using Eppendorf tubes. WGA biofunctionalization of polysilicon barcodes was achieved in four steps:

(i) Surface activation. The polysilicon surface of the barcodes was first activated by treatment with piranha solution H$_2$SO$_4$·H$_2$O$_2$ (7:3, 1 ml) for 1 h; after this time, the barcodes were washed with deionized water (3 × 1 ml) followed by centrifugation at each step (2 min at 13 000 rpm) to eliminate the supernatants. Next, 1 ml of basic solution NH$_4$OH·H$_2$O$_2$·H$_2$O (1:1:5, 1 ml) for 30 min was added in order to ensure the hydroxylation of the surface. Finally, the barcodes were washed with deionized water (3 × 1 ml) and absolute ethanol (3 × 1 ml), with centrifugation in every step to eliminate the supernatant.

(ii) Silane immobilization. 10 μl of acetic acid buffer (pH 5.2) were added to 180 μl of absolute ethanol and 10 μl of triethoxysilaneundecanal (TESUD; ABCR, Germany), corresponding to a final TESUD concentration of 135 mM. The polysilicon barcodes were immersed for 3 h at room temperature in this solution. Next, they were washed with absolute ethanol (3 × 1 ml) followed by centrifugation after every step to eliminate the supernatant, and finally they were placed into an oven at 90°C for 1 h.

(iii) Protein immobilization. The terminal aldehyde groups reacted with WGA amine groups under reductive conditions by adding a WGA (Invitrogen, USA) solution in phosphate-buffered saline (PBS; 0.4 ml, 35 μg/ml) to the previously aldehyde covered barcodes, in the presence of a PBS solution of sodium cyanoborohydride (5 mM, 50 μl; Sigma). The mixture was kept at 4°C overnight.

(iv) Chemical blocking. To cover the unreacted aldehyde groups on the surface, a PBS solution of 2-(2-aminooethoxy)ethanol (15 mM, 50 μl; Sigma) was added. After 20 min, the barcodes were centrifuged, and washed with autoclaved PBS (3 × 1 ml), followed by centrifugation after every step to eliminate the unspecific protein adsorption.

The biofunctionalized barcodes were kept and stored as a suspension in autoclaved PBS in the fridge at 4°C.

Collection of mouse embryos

Mouse females of the hybrid strain B6CBAF1 (C57BL/6J × CBA/J), 8–12 weeks old, were used as embryo donors. Ovulation induction was induced by intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (Intervet, Spain). After 48 h, a second injection of 5 IU of human chorionic gonadotrophin (hCG; Divasa-Far marvic, Spain) was administered and the females were mated with B6CBAF1 males. Pronuclear embryos were collected from the ampulla 25 h after hCG administration and incubated during 5–10 min at 37°C in HEPES-buffered potassium simplex optimized medium (H-KSOM; Biggers et al., 2000) supplemented with 156 U/ml of hyaluronidase (Sigma, Spain) for dispersion of the cumulus cells. Denuded embryos were washed twice in fresh H-KSOM, and embryos with two pronuclei and a good morphology were incubated in KSOM(aa) culture medium containing both essential and non-essential amino acids at 37°C in a 5% CO$_2$ atmosphere until tagging.

Embryo tagging, and culture and monitoring

Pronuclear embryos were tagged by the attachment of 10 biofunctionalized barcodes to the outer surface of their ZP. First, a drop of PBS containing the biofunctionalized barcodes was placed in a dish and, by micromanipulation, groups of 10 barcodes were transferred to separate drops of H-KSOM, distributing them strategically in two rows at the bottom of each drop (five barcodes per row) at the bottom of a manipulation medium drop to ensure, after rolling an embryo over them, an equidistant distribution of the barcodes around the ZP surface. Two different focal planes of the same embryo with a total of 10 barcodes attached to the outer surface of its ZP.

The fabrication process has been previously described (Fernández-Rosas et al., 2009; Novo et al., 2011).
were analyzed by the group of control non-tagged embryos. Six to 12 embryos were loaded for 72–96 h. Thawed embryos were monitored every 24 h and therefore the embryo could be successfully identified, retaining) and the number of developed embryos in which at least one barcode was remaining attached to the ZP of the developed embryos (mean ± SEM). A total of 140 pronuclear-stage mouse embryos, divided in six time points, embryo identification was successful in 96.5% of the total analyses realized (n = 486). Moreover, it is important to point out that even the small number of non-identifiable embryos could be finally identified simply by rotating them to allow the correct orientation of at least one of their barcodes.

Embryo freezing and thawing
Tagged embryos which had cleaved to the 2-cell stage and retained the 10 barcodes attached to the ZP, were frozen using a slow freezing protocol (Costa-Borges et al., 2009; Novo et al., 2011), in parallel to a group of control non-tagged embryos. Six to 12 embryos were loaded per straw. One to 7 days after cryopreservation embryos were thawed, transferred to drops of KSOM(aa) and cultured at 37°C in a 5% CO₂ atmosphere for 72–96 h. Thawed embryos were monitored every 24 h and development rates, mean barcode retention and identification rates were assessed. In addition, barcode release rate after hatching was also determined.

Statistical analysis
All experiments were repeated at least three times on separate days and the results achieved were pooled. In vitro and in vivo development rates were analyzed by the χ² test or Fisher’s exact test and the identification rates by ANOVA. The values of barcode retention were compared using Kruskal–Wallis and Dunn tests. A P-value of <0.05 was considered statistically significant.

Results

In vitro development of barcode-tagged embryos
To test the validity of the new embryo tagging system, tagged embryos were allowed to develop in vitro and were monitored every 24 h. A total of 140 pronuclear-stage mouse embryos, divided in six experiments, were tagged with 10-WGA-biofunctionalized barcodes each (Fig. 2). Ideally each embryo should have been tagged with various copies of the same barcode number to simulate an eventual situation in a clinical setting in which all embryos from the same patient or couple would be tagged with a unique barcode number. However, this was not possible at this stage as barcodes were fabricated in all possible combinations in a single silicon wafer and they were mixed upon release.

After 96 h of in vitro culture 90% (n = 126) of the tagged embryos achieved the blastocyst stage (Fig. 3), a rate of development equivalent to that of the control non-tagged group (88.3%; n = 60). Regarding barcode retention (Table I), it should be noted that when tagged embryos were transferred from the drops of manipulation medium where tagging was performed to the drops of culture medium (0 h of culture), 16 of the tagged embryos (11.4%) lost some of the barcodes (maximum loss of three barcodes per embryo). A few additional barcodes were progressively lost during embryo culture and, in fact, after 48 h of culture the mean retention value, despite being high (9.75 ± 0.05 barcodes per embryo), became significantly lower than the initial number of barcodes attached per embryo (10; Table I). Another significant decrease in the mean retention value was observed between 48 and 96 h (Table I). However, in spite of these losses, after 96 h of in vitro culture 65.9% of the embryos still retained the 10 barcodes initially attached and the mean retention value was as high as 9.56 ± 0.06 barcodes per embryo. More important, 100% of the tagged embryos maintained at least seven barcodes attached to their ZP.

Identification rates, assessed in 100 tagged embryos, were high and similar at all time points analyzed (94.8–98.0%; Table II). As expected, the total identification rates decreased as the number of barcodes that remained attached per embryo decreased, though significant differences were only observed in embryos with less than nine barcodes. Regardless of the number of barcodes attached per embryo and the culture time point, embryo identification was successful in 96.5% of the total analyses realized (n = 486). Moreover, it is important to point out that even the small number of non-identifiable embryos could be finally identified simply by rotating them to allow the correct orientation of at least one of their barcodes.

Embryos that reached the blastocyst stage by 96 h were kept in culture for an additional 24 h to assess the fate of the barcodes after embryo hatching. As expected, all the barcodes remained attached to the ZP and a barcode release rate of 100% was achieved in the hatched embryos (Fig. 4).

Full-term development of barcode-tagged embryos
In a second set of experiments, the in vivo development of the tagged embryos was assessed and compared with that of a group of control non-tagged embryos. A total of 105 2-cell embryos tagged with 10 barcodes at the pronuclear stage and of 101 2-cell control embryos were transferred into the oviducts of 10 and 11 recipient females, respectively. Surprisingly, full-term development of the tagged embryos (78/105, 74.3%) turned out to be significantly higher than that of the control non-tagged ones (58/101, 57.4%; P-value = 0.0162). On the other hand, all surrogate females and their offspring were apparently healthy and all the obtained pups showed an apparently normal development.
Cryopreservation of barcode-tagged embryos

A last set of experiments was performed to test the validity of the embryo tagging system after a cryopreservation process. To this aim, embryos were tagged with 10 barcodes at the pronuclear stage, cultured during 24 h and cryopreserved at the 2-cell stage ($n = 84$), in parallel to a group of control non-tagged embryos ($n = 75$). After thawing, no significant differences in development rates were observed between the two groups, achieving an equivalent blastocyst rate after 72 h of culture (84.5% tagged; 81.3% control). Most of the embryos (76.5%; Table III) lost at least one barcode during the cryopreservation process, being the loss of one to three barcodes the most common and five barcodes the maximum loss. Thus, mean retention values decreased to 8.52 ± 0.14 after thawing (0 h of culture), but then stabilized during the 72 h of culture (Table III). At the end of the culture, the mean barcode retention value in frozen–thawed embryos (8.38 ± 0.14; Table III) was significantly lower than that achieved in non-cryopreserved embryos (9.56 ± 0.06; Table I) and the modal retention value decreased from 10 (Table I) to 8 (Table III).

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

<table>
<thead>
<tr>
<th>Number of barcodes per embryo</th>
<th>Tagged embryos</th>
<th>Developed embryos with barcodes (%)</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>140</td>
<td>124 (88.6)</td>
<td>121   (88.3)</td>
<td>108   (80.0)</td>
<td>93    (71.5)</td>
<td>83    (65.9)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>13 (9.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>2 (1.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1 (0.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean retention ± SEM*</td>
<td>10*</td>
<td>9.86 ± 0.04±b</td>
<td>9.85 ± 0.04±b</td>
<td>9.75 ± 0.05±bc</td>
<td>9.64 ± 0.06±d</td>
<td>9.56 ± 0.06±d</td>
<td></td>
</tr>
</tbody>
</table>

SEM, standard error of the mean.

*Mean number of barcodes attached per embryo.

**Different superscripts denote significant differences among mean retention values at different time points ($P < 0.05$).
Identification rates of the frozen–thawed tagged embryos were similar at all the time points analyzed (87.7–94.4%; Table IV). As observed in non-cryopreserved embryos, the identification rate was directly related to the number of barcodes that remained attached to the ZP, decreasing as the number of barcodes decreased. But the decrease was significant only when less than seven barcodes were present (Table IV). Consequently, the global percentage of successful identification processes out of the total performed during the culture of the cryopreserved embryos (92.4%, \( n = 381 \); Table IV) was significantly lower than that of the non-cryopreserved group (96.5%, \( n = 486 \); Table II). However, because all the barcodes maintained their integrity after freezing and thawing and all the embryos retained at least five barcodes after cryopreservation, an identification rate of 100% could be achieved simply by rotating the

Table II  Identification rates of tagged embryos at different time points during in vitro culture.

<table>
<thead>
<tr>
<th>Number of barcodes per embryo</th>
<th>Developed embryos successfully identified (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>10</td>
<td>88/88 (100)</td>
</tr>
<tr>
<td>9</td>
<td>8/9 (88.9)</td>
</tr>
<tr>
<td>8</td>
<td>1/2 (50.0)</td>
</tr>
<tr>
<td>7</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>97/100 (97.0)</td>
</tr>
</tbody>
</table>

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

*Values with different superscripts significantly differ (\( P < 0.05 \)).

Figure 4 Barcode release after blastocyst hatching. Two different focal planes (A and B) of a hatched blastocyst free of barcodes and of the corresponding empty ZP with the 10 barcodes attached to its outer surface.

Table III  Barcode retention after thawing and during culture of frozen-thawed tagged embryos.

<table>
<thead>
<tr>
<th>Number of barcodes per embryo</th>
<th>2-cell embryos cryopreserved</th>
<th>Developed embryos with barcodes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>10</td>
<td>19 (23.5)</td>
<td>16 (21.6)</td>
</tr>
<tr>
<td>9</td>
<td>27 (33.3)</td>
<td>26 (35.1)</td>
</tr>
<tr>
<td>8</td>
<td>18 (22.2)</td>
<td>19 (25.7)</td>
</tr>
<tr>
<td>7</td>
<td>12 (14.8)</td>
<td>10 (13.5)</td>
</tr>
<tr>
<td>6</td>
<td>4 (5.0)</td>
<td>2 (2.7)</td>
</tr>
<tr>
<td>5</td>
<td>1 (1.2)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>( \leq 4 )</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

SEM, standard error of the mean.

*Mean number of barcodes attached per embryo.

Different superscripts denote significant differences among mean retention values at different time points (\( P < 0.05 \)).
non-identifiable embryos until at least one of the remaining barcodes was properly oriented for its reading.

As in the first set of experiments, when blastocysts were kept in culture until hatching a barcode release rate of 100% was achieved, as all the barcodes remained attached to the empty ZP of the hatched embryos.

**Discussion**

Our group is working on the development of new methods for the identification of reproductive samples, based on their direct tagging with polysilicon barcodes, to ensure their correct traceability and, therefore, minimize the risk of sample mix-ups in ARTs. A method presented in the present work, in which WGA-biofunctionalized barcodes are attached to the outer surface of the ZP, was conceived to overcome the main limitations of our previously reported method, in which barcodes were microinjected into the perivitelline space. Namely, these limitations were the need for micromanipulation techniques to tag each individual embryo, and the adhesion of the barcodes to the embryo surface after hatching. Whereas the latter has been successfully overcome with the new embryo tagging method, as will be discussed later, the former has been only partially solved. Thus, embryo tagging can now be performed under the stereoscopic microscope only with a mouth- or hand-controlled aspiration system. The process is simple and fast, requiring only \( \sim 20 \) s to attach 10 barcodes to each embryo. However, micromanipulation is still required in a previous step to arrange the barcodes at the bottom of the manipulation drop so that when the embryo is rolled over the barcodes these become uniformly distributed around the ZP. This uniform distribution will increase the probability that at least one of the barcodes is properly oriented for reading without having to rotate the embryo and, therefore, that the embryo can be successfully identified without manipulation. In fact, this is also the reason why several barcodes, and not just one, were used to tag each embryo. Even though manual eye reading of the barcodes was performed in the present study for embryo identification, we are currently testing a computer program developed specifically for the automatic reading of the barcodes. The use of this software will allow a faster and more reliable reading of the barcodes just by placing the tagged embryos under the inverted microscope and capturing an image of one of its barcodes. In this situation, having to rotate each embryo for proper barcode orientation for reading has to be avoided. The attachment of more than 10 barcodes per embryo could increase the probability that, independently of the embryo orientation, at least one of the barcodes could be successfully read. Alternatively, a new approach to ensure the uniform distribution of the barcodes around the ZP without the need for micromanipulation could be devised, and our efforts are now focused in this direction.

To test the validity of the new tagging system, in vitro culture of tagged embryos was first carried out. No effect of the attached WGA-biofunctionalized barcodes on the developmental potential of the tagged embryos up to the blastocyst stage was observed, as expected according to previous studies by our group. On the other hand, polysilicon microparticles and barcodes did not affect cell viability when internalized into human macrophages by phagocytosis (Fernández-Rosas et al., 2009, 2010), nor did they affect developmental potential when microinjected into the perivitelline space or even into the cytoplasm of mouse embryos (Fernández-Rosas et al., 2010; Novo et al., 2011). On the other hand, even though free WGA lectin has showed embryo toxicity when used as supplement of culture media at different concentrations (Menino et al., 1989), we have demonstrated that its covalent attachment to the barcodes surface by an efficient biofunctionalization protocol prevents its contact with the embryo cell membrane and, therefore, its toxic effects (unpublished results). In fact, the efficiency of the biofunctionalization protocol used in the present study has been proved not only by the absence of toxic effects of the WGA-biofunctionalized barcodes, but also by the high barcode retention mean values achieved (9.56 \( \pm \) 0.06 barcodes per embryo at 96 h of culture). The detachment of some of the barcodes in \( <35\% \) of the tagged embryos during \( in vitro \) culture could be attributed to a slight weakening of the WGA–ZP binding resulting from the modifications occurring in the ZP during early embryo development (Vanroose et al., 2000).

Despite some barcodes detached from the ZP, all of the tagged embryos retained at least seven barcodes. This allowed to achieve a high identification rate (96.5%), similar to the one obtained with the previously reported embryo tagging system (97%) based on the microinjection of the barcodes into the perivitelline space (Novo et al., 2011). As pointed out earlier, this identification rate was obtained.

### Table IV Identification rates of frozen–thawed tagged embryos at different time points during \( in vitro \) culture.

<table>
<thead>
<tr>
<th>Number of barcodes per embryo</th>
<th>2-cell embryos cryopreserved</th>
<th>Developed embryos successfully identified (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>80/84 (95.2)</td>
<td>0 h: 18/19 (94.7)</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>24 h: 15/16 (87.5)</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>48 h: 15/15 (100)</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>72 h: 15/15 (100)</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Total: 143/149 (96.0)*</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

\(^a\)–c Values with different superscripts significantly differ \( \left( P < 0.05 \right) \).
without embryo manipulation at the time of reading. In fact, because none of the tagged embryos lost all of the barcodes during culture, and identification rate of 100% could have been achieved simply by rotating the non-identifiable embryos until one of the barcodes became properly oriented for reading.

The system reported here is designed to tag embryos exclusively during their manipulation and in vitro culture in the laboratory, so that each individual embryo can be identified and tracked at any point of the ART procedure. However, the embryos should be free of the barcodes before they implant into the maternal uterus. As previously mentioned, one of the limitations of the first reported embryo direct tagging system was the adhesion of the barcodes to the embryo cells surface after blastocyst hatching (Novo et al., 2011). This limitation has been successfully overcome with the new tagging system, as all the barcodes remained attached to the empty ZP after embryo hatching and 100% of the hatched embryos were totally free of barcodes. In this situation, implantation and post-implantation development probably occurs without any contact between the embryos and the barcodes. In fact, the high offspring rates after transfer of the tagged embryos into recipient females confirm that barcodes attached to the ZP do not adversely interfere with hatching, implantation and full-term development. The higher offspring rate of tagged embryos when compared with non-tagged ones was unexpected and we currently do not have any explanation for this fact. On the other hand, the fate of the barcodes in the female body is unknown and, because of their small size, their localization inside the female reproductive track by histological studies would not be possible. As barcodes are not biodegradable, one can hypothesize that when the ZP is degraded by the uterine zona-lytic activity (Lin et al., 2001) the barcodes are released into the uterus and they might be eventually removed from the female body with the decidua shedding after parturition (Salamonsen, 2003).

Embryo cryopreservation is a common procedure at fertility clinics. As it has been reported that cryopreservation causes physical ZP damage (Van den Abbeel and Van Steirteghem, 2000) and induces biochemical changes related to the secondary structure of proteins and carbohydrate residues (Bogliolo et al., 2012), the effectiveness of the embryo tagging system presented here, which is based on the recognition and binding of WGA to the ZP glycoproteins, could be significantly altered during embryo cryopreservation. Our results showed that cryopreservation had no detrimental effects on the in vitro developmental potential of tagged embryos. However, frozen–thawed embryos showed a more evident detachment of the WGA–biofunctionalized barcodes at the beginning of the culture in comparison with fresh–cultured embryos and, consequently, the mean barcode retention values at the end of the culture period were significantly lower in cryopreserved than in fresh tagged embryos. These results suggest that either the more extensive manipulation of the cryopreserved embryos before culture (various transfers of embryos from one solution to another during freezing and thawing procedures) in comparison with the fresh embryos or, more probably, the physical and biochemical modifications that the ZP undergoes during embryo cryopreservation may impose a significant stress on the WGA–ZP binding, which results in the detachment of some barcodes. As a consequence, the global identification rate of cryopreserved embryos was significantly reduced when compared with that of the fresh embryos. However, the identification rate was still higher than 92% and, as all the embryos retained at least five barcodes, successful identification of all the tagged embryos would have been possible simply by rotating the embryos.

In conclusion, both in vitro and in vivo development of the tagged embryos were unaffected by the presence of the barcodes, and their identification rate during the in vitro culture period was high and equivalent to that achieved with our first reported embryo tagging approach, indicating that the attachment of biofunctionalized barcodes to the outer surface of the ZP is a valid alternative to the microinjection of barcodes into the perivitelline space for embryo identification purposes and overcomes its limitations (Novo et al., 2011). Moreover, the effectiveness of the tagging system is not significantly decreased after embryo cryopreservation. These results provide a proof of concept for a direct embryo tagging system using WGA-biofunctionalized polysilicon barcodes, and the system is currently being tested for its application in human oocytes and embryos donated for research, and in bovine embryos.

Authors’ roles

L.B., C.N., J.S. and E.I. conceived the study and, together with S.N., designed the experiments; J.A.P. designed the barcodes; R.G.M. and S.D. fabricated the barcodes; O.P., J.S. and L.P.G. biofunctionalized the barcodes; S.N. performed the experiments and, together with E.I., analyzed the data and wrote the manuscript; all authors critically revised the manuscript and approved the final version.

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Conflict of interest

None declared.

References

Barcode attachment to the zona to tag embryos


