Etiquetaje directo de ovocitos y embriones

Memoria presentada por Sergio Novo Bruña para optar al grado de Doctor por la Universitat Autònoma de Barcelona dentro del programa de doctorado de Biología Celular

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CERTIFICAN

Que Sergio Novo Bruña ha realizado bajo su dirección el presente trabajo de investigación que lleva por titulo "Etiquetaje directo de ovocitos y embriones" para optar al grado de Doctor por la Universitat Autònoma de Barcelona.

Que este trabajo se ha llevado a cabo en la Unitat de Biologia Cel·lular del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona.

Y para que así conste, firman el presente certificado.

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A mis padres…

…mis mejores maestros

y a mis hermanos…

…espejos donde mirarme

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1. INTRODUCCIÓN

1.1. Identificación y trazabilidad de muestras biológicas reproductivas

En la sociedad actual se obtiene un elevado número de muestras biológicas, tales como órganos, tejidos, células o fluidos, que inevitablemente han de compartir espacio y tiempo con muestras de su misma índole pero de origen distinto. Por ello, la correcta identificación de la muestra es indispensable para poder realizar su seguimiento desde la obtención hasta su procesamiento, preservación, almacenamiento y distribución. La preservación de la trazabilidad de cada una de las muestras biológicas obtenidas es de suma importancia, ya sea por su valor material (muestras biológicas de animales con pedigrí) o terapéutico (muestras biológicas humanas destinadas a trasplantes, transfusiones, implantaciones, etc.).

El real decreto 1301/2006, del 10 de Noviembre, establece unas normas en el control de la calidad y seguridad para la donación, la obtención, la evaluación, el procesamiento, la preservación, el almacenamiento y la distribución de células y tejidos humanos. El artículo 32 de este real decreto, relacionado con la trazabilidad, establece que un sistema de rastreo de origen a destino de cualquier célula y tejido obtenidos ha de estar asociado a un sistema de codificación que recoja la información relevante de la muestra (identificación de la donación, del establecimiento y del producto). La identificación de una muestra se logra con la vinculación a esta de un elemento identificador único y exclusivo, en definitiva, una etiqueta. El etiquetado ha de permitir identificar claramente las muestras en todas las fases del procedimiento establecido para cada tipo de muestra. El etiquetado de muestras de gran tamaño, como órganos o tejidos, muestras fluidas (principalmente derivados sanguíneos y orina) y muestras microscópicas únicas (por ejemplo gametos y embriones) está basado en la vinculación de un elemento identificador exclusivo al recipiente que las contiene. Si una muestra no precisa un procesamiento complejo previo a su análisis o implantación, la trazabilidad es segura usando estos sistemas. En cambio, si la muestra ha de ser procesada, la preservación de su correcta trazabilidad se complica puesto que el procesamiento suele implicar uno o más cambios de recipiente. En su gran mayoría, los errores de trazabilidad o de identificación de muestras (*mix-ups*) están ligados a errores

humanos en los que el paciente no ha sido correctamente vinculado con la muestra correspondiente o tratamiento específico (National Patient Safety Agency, 2004). Los incidentes de *mix-ups* ocurren como resultado de errores de identificación que pueden darse en distintos procesos médicos, incluyendo análisis clínicos en laboratorios, prescripción y administración de medicamentos, cirugías y transfusiones sanguíneas (Plebani et al., 1997; Bates et al., 2001; Stainsby et al., 2004).

Las muestras biológicas reproductivas (gametos y embriones) que se utilizan en las técnicas de reproducción asistida (TRAs) son un ejemplo de muestra microscópica confinada en un recipiente etiquetado para su identificación. Se trata de muestras que han de seguir numerosos y complejos procesamientos, lo que implica que su trazabilidad sea altamente susceptible a experimentar el denominado "error humano". Los puntos más críticos en el control de la trazabilidad de este tipo de muestras están relacionados con la identificación de la complementariedad en el momento de la fecundación (entre los ovocitos de una paciente y la muestra seminal correspondiente) y en el momento de la transferencia (entre los embriones y la paciente receptora).

Todo sistema de identificación ha de permitir mantener la muestra correctamente identificada durante todos su procesado y permitir trazar su recorrido con la menor intervención humana posible.

1.1.1. Puntos críticos en la trazabilidad durante el procesamiento de muestras biológicas reproductivas

La aplicación de las TRAs implica la obtención y procesamiento de mayoritariamente tres tipos de muestras biológicas: ovocitos, espermatozoides y embriones. El tiempo de vida en fresco en el laboratorio de cada una de estas muestras es reducido, del orden de horas o días, mientras que el tiempo de preservación y almacenaje en tanques de nitrógeno es indeterminado, pudiendo llegar a ser del orden de años. Los procesos a los que se someten cada una de las

muestras son distintos, aunque en el caso de los ovocitos y espermatozoides suelen converger en un procesamiento conjunto.

El riesgo de *mix-up* existe cuando muestras de distintos orígenes son procesadas simultáneamente o comparten un almacenaje común (tanque de nitrógeno, incubadora, placa de cultivo). En estas circunstancias, los procesos que requieren la transferencia de las muestras de un recipiente a otro son los puntos más críticos a la hora de preservar su correcta trazabilidad. Errores en el reconocimiento de la identificación del recipiente precedente o posterior durante un procesado, ya sea por un error en la asociación del etiquetaje con el recipiente o en la lectura de la etiqueta identificativa, pueden dar lugar a *mix-ups*. Los cambios de recipiente a los que se somete cada una de las muestras reproductivas en función de su tipología son más o menos frecuentes (representados en la Figura 1):

- Muestra seminal: la muestra de semen se deposita en un recipiente para la recogida de muestras clínicas en estado fluido. Si se trata de una muestra válida para su uso con fines reproductivos, puede ser utilizada para una inseminación artificial, directamente criopreservada o procesada para aislar la fase móvil (mediante gradientes de densidad o *swim-up*). Este procesamiento consiste en traspasar un volumen de la muestra seminal del recipiente de recogida al recipiente de procesamiento (tubo cónico con las soluciones correspondientes). Después se recupera el volumen que contiene la fase móvil de la muestra y se destina a la fecundación o a su almacenamiento por criopreservación. La primera opción requiere la transferencia de cierto volumen de la muestra procesada a otro recipiente (placa) donde se llevará a cabo la fecundación ya sea mediante fecundación *in vitro* (FIV) convencional o inyección intracitoplásmatica de espermatozoides (*IntraCytoplasmic Sperm Injection*, ICSI). Si la opción es la criopreservación la muestra es almacenada en un recipiente específico (pajuela o criotubo). Una vez descongelada, se elimina el crioprotector en un nuevo recipiente, si la muestra ya había sido procesada se utiliza para la

fecundación y si no, se procesa para finalmente utilizarla para la fecundación.

- Ovocitos: el liquido folicular es recolectado mediante punción folicular en unos tubos que posteriormente son vertidos en una placa para, bajo un microscopio estereoscópico, buscar los complejos cumulus-ovocito (*Cumulus-Oocyte Complex*, COC). Una vez recolectados, si el ovocito requiere maduración, los COCs se colocan en una placa con medio de maduración donde permanecen unas horas hasta el momento en el que son fecundados con la muestra seminal correspondiente. Como anteriormente se ha mencionado, el ovocito puede ser fecundado mediante FIV convencional o ICSI. Para el primer procedimiento, es necesario mantener las células del cumulus, y únicamente hay que colocar los ovocitos en medio de fecundación junto con una concentración adecuada de espermatozoides en un nuevo recipiente. En cambio para practicar ICSI se requiere la denudación previa del ovocito, ya sea mecánicamente o con la ayuda de proteasas, en un nuevo recipiente. Una vez denudado, el ovocito se traslada a una gota de medio de manipulación en una nueva placa, donde en otra gota se encuentra la muestra seminal complementaria. Se selecciona un espermatozoide y se introduce en el interior del ovocito mediante técnicas de micromanipulación. Si es necesaria una activación partenogenética, esta se realiza en otro recipiente donde el ovocito inyectado pasa por varias soluciones. Finalmente, el ovocito fecundado es colocado en un nuevo recipiente donde será cultivado
- Embriones: después de la comprobación de la fecundación, los embriones obtenidos son transferidos a un nuevo recipiente para ser cultivados. En función de si el cultivo embrionario se realiza con una serie

de medios secuenciales o con un medio global, los embriones tendrán que ser transferidos a nuevos recipientes o no hasta su transferencia o criopreservación. Cuando se decide criopreservar los embriones, estos han de cambiar de recipiente varias veces durante el procesado hasta su transferencia al soporte de criopreservación.

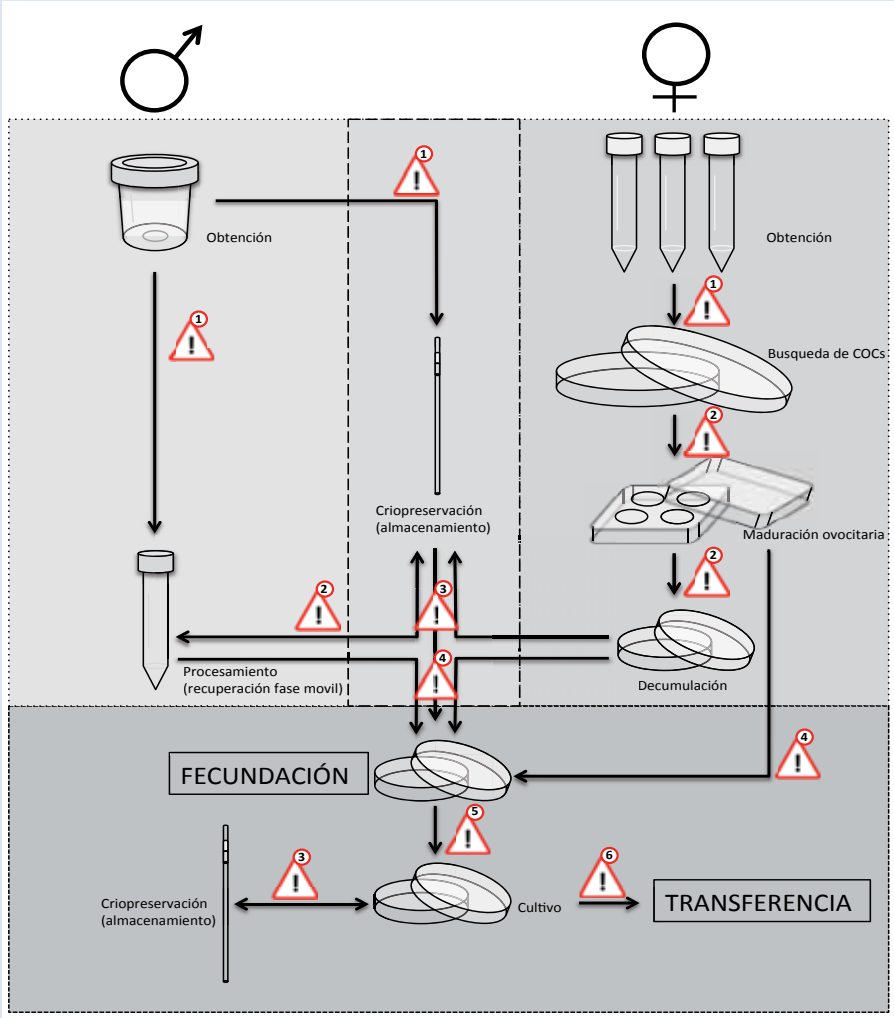

Figura 1. Puntos críticos y tipos de error en la trazabilidad de las muestras reproductivas durante los procesos a los que generalmente son sometidas durante un ciclo de TRA. Error 1: identificación del recipiente de obtención; Error 2: trazabilidad durante el procesado; Error 3: identificación del recipiente de criopreservación; Error 4: complementariedad entre muestras para fecundación; Error 5: trazabilidad en cultivo; Error 6: complementariedad entre muestra y receptora.

Los procedimientos a los que son sometidas las muestras reproductivas, arriba descritos, son varios, diversos y complejos. Para preservar la correcta trazabilidad de todos estos tipos de muestras reproductivas durante sus procesamientos se utilizan sistemas de identificación. Estos sistemas pueden dividirse en sistemas de identificación para muestras humanas y sistemas de identificación para muestras de animales de renta.

1.1.2. Identificación y trazabilidad de muestras reproductivas humanas

Las TRAs fueron desarrolladas con el fin de lograr la reproducción de parejas con problemas para la concepción de forma natural. Desde que se establecieran en el campo clínico en 1978 hasta la actualidad, las TRAs han experimentado una continua progresión en el número de casos clínicos registrados cada año. Según estimaciones del 2010 de la Organización Mundial de la Salud (Mascarenhas et al., 2012), existen alrededor de un 1,9% y un 10,5% de casos de infertilidad primaria y secundaria, respectivamente. Europa lidera la práctica de TRAs con el 71% de los ciclos registrados en el mundo (sin tener en cuenta Asia por falta de datos). Los últimos datos conocidos cifran los ciclos anuales realizados en 537.463 en Europa (datos 2009; Ferraretti et al. 2013), 147.264 en Estados Unidos (datos 2010; CDC et al., 2012) y 66.347 en Australia y Nueva Zelanda (datos 2011; Macaldowie et al., 2013)(Figura 2). En España el número de ciclos en 2009 fue de 54.266 (datos 2009; Ferraretti et al. 2013) y, según la Asociación Nacional de Clínicas de Reproducción Asistida cada año aumentan los casos de esterilidad en 30.000 casos.

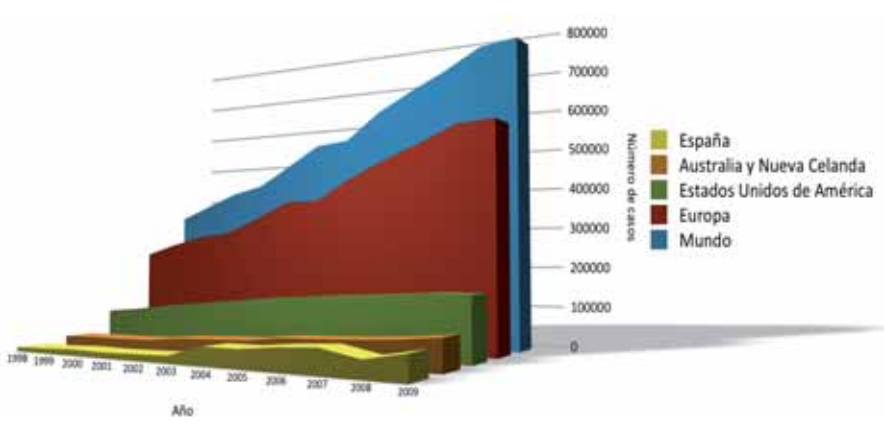

Figura 2. Evolución del número de ciclos de TRAs registrados desde el 1998 al 2009. Datos de Europa y España (A.P. Ferraretti et al. 2013), Estados Unidos (CDC et al., 2012) y Australia y Nueva Zelanda (Macaldowie et al., 2013).

Este gran volumen de pacientes requiere de una gran infraestructura sanitaria, detrás de la cual hay grandes inversiones de capital. Pese a ello, actualmente es imposible individualizar totalmente el proceso y destinar un lugar de trabajo, almacenamiento e incubación único para cada muestra, lo que implica la simultaneidad de ciclos de reproducción asistida independientes. Para poder distinguir entre los distintos casos han de seguirse estrictos protocolos de control de la trazabilidad e identificar cuidadosamente el material reproductivo de un mismo caso, tanto los gametos (ovocitos y espermatozoides) como los embriones derivados de estos. Ahún así, la práctica de las TRAs no está exenta de errores humanos y varios casos de *mix-ups* entre material reproductivo de distintos pacientes y han sido descritos en centros de todo el mundo. Uno de los primeros casos, documentado en 1972 y detectado tras el nacimiento de un bebé de piel negra de una mujer caucásica, fue debido a un *mix-up* de la muestra seminal a utilizar en el proceso de inseminación artificial al que se sometió la paciente (Kleegman, 1972). De hecho 66% de los *mix-ups* documentados son debidos a errores de identificación de la muestra seminal, mientras que el 44% restante se atribuyen a errores de identificación de los embriones. Más de la mitad de los *mix-ups* documentados han dado lugar a nacimiento, y de ellos aproximadamente la

mitad han sido detectados por características fenotípicas (Figura 3). Aunque el número de casos documentados es relativamente bajo teniendo en cuenta el número de ciclos que se realizan (la *Human Fertilization and Embryology Authority* establece una frecuencia de *mix-ups* en el 0,04% de los ciclos realizados; Shaikh, 2009), seguramente han ocurrido más casos, que han pasado desapercibidos al no tener factores fenotípicos diferenciales para se detectados (Spriggs, 2003), ya que no se suelen hacer pruebas de paternidad en concepciones logradas mediante TRAs.

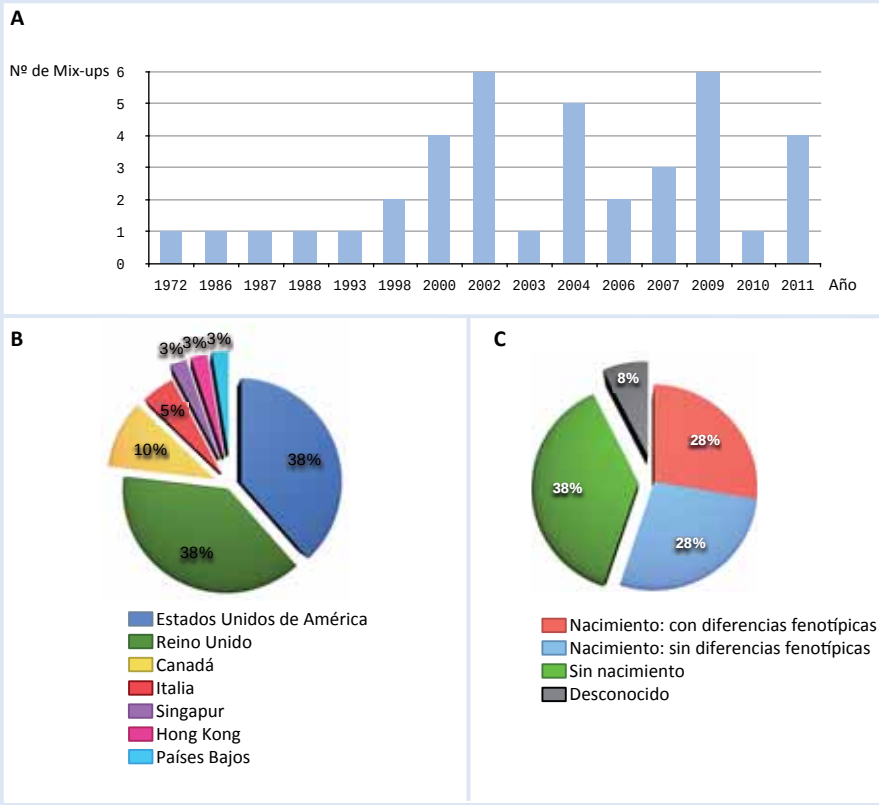

Figura 3. Análisis de los casos de *mix-ups* documentados en la práctica de las TRAs. (A) Número de *mix-ups* detectados cada año **(39 casos en total)**. **(B)** Distribución por países de los casos de *mix-ups* detectados. **(C)** Resultado de los *mix-ups* (Kleegman 1972; Dyer, 2002 y 2004; BioNews Associate Press, 2004ª y 2004b; Christoffersen, 2004; Ford et al., 2004; BioNews Associate Press, 2005; Bender, 2006; Blackburn-Starza, 2006; Kabir Banu az-Zubair, 2006; Roberts, 2007; Mallon, 2009; Raper, 2009; Stevens, 2009a, 2009b y 2009c; Savage et al., 2010; Ahmad, 2011; Fatimathas, 2011; Photopoulos, 2011; Sheldon, 2011; Fox News Associate Press, 2012; Sheppard, 2012; Long, 2013).

Las repercusiones de estos sucesos pueden afectar a más de una pareja de pacientes y conllevar la destrucción de muestras (Toft, 2004), disputas de paternidad (Dyer, 2003; Ford et al., 2004; Kabir Banu az-Zubair, 2006), adopciones postparto (Morell et al., 2010; Savage et al., 2010) o abortos (Dyer, 2003). Por otro lado, estos incidentes siempre repercuten legal y económicamente en los centros y profesionales responsables del error, repercusiones que van desde indemnizaciones millonarias (NBCNews Associated press, 2004) a inhabilitaciones profesionales (Long, 2013) y el correspondiente daño a la imagen del centro frente a sus presentes y futuros clientes.

Si bien los errores de identificación del material reproductivo pueden ocurrir en cualquier momento del transcurso de un ciclo de reproducción asistida, los puntos más críticos son el momento de la fecundación (uso de ovocitos o espermatozoides que no corresponden a los pacientes), los cambios de placa (etiquetado incorrecto de las placas cuando los ovocitos o embriones se cambian de medio) y la transferencia embrionaria (errores en la concordancia entre el origen de los embriones y la paciente a la que se transfieren).

Los profesionales de las TRAs, conscientes de la importancia de evitar al máximo estas equivocaciones, y a través de asociaciones como la *European Society for Human Reproduction & Embryology* (Gianaroli et al., 2000), la Federación Latinoamericana de Sociedades de Esterilidad y Fertilidad (Elder et al., 2003) y la *Human Fertilization and Embryology Authority* (HFEA; Code of practice, 2003) propusieron, en sus guías de buenas prácticas para laboratorios de reproducción asistida, que todo material de laboratorio que contenga muestras biológicas fuera correctamente etiquetado y que toda acción realizada en el transcurso de un caso clínico fuera comprobada por una segunda persona que verifique el procedimiento o lo corrija en su defecto (doble chequeo).

El sistema de etiquetaje más utilizado actualmente es el manual, basado en la identificación de todo el material destinado a un caso clínico mediante la escritura en su superficie plástica de elementos identificativos únicos y exclusivos como

pueden ser el nombre del paciente o número de registro y color del marcaje. La aplicación del doble chequeo en cada uno de los momentos de etiquetaje del material de laboratorio y de toma de decisión del destino del material biológico (cambio de recipiente, transferencia de embriones, etc.) supone un aumento del número de embriólogos destinados a cada caso clínico, lo que se traduce en un aumento del coste del procedimiento. Por otro lado, la eficacia del doble chequeo ha sido cuestionada, ya que su aplicación puede derivar en errores por automatización involuntaria (Toft et al., 2005). Además, debido a que el embriólogo ha de ser interrumpido de sus tareas cuando se le reclama para un doble chequeo, este sistema puede incrementar el riesgo de error al crear continuas distracciones e interrupciones al propio trabajo del embriólogo (Brison et al., 2004; Mortimer et al., 2005)

Consciente de las limitaciones que plantea el control de la trazabilidad mediante el etiquetaje manual del material de laboratorio y el doble chequeo, la HFEA, una de las asociaciones más concienciada con el control y seguridad de la trazabilidad en la aplicación de las TRAs en el Reino Unido, promueve actualmente el uso de sistemas basados en la identificación del material de laboratorio utilizado para cada caso clínico mediante etiquetas electrónicas asociadas a sistemas de lectura automatizados, encargados de supervisar la correcta trazabilidad del material biológico. Los sistemas desarrollados hasta el momento con este fin se basan en etiquetas autoadhesivas que deben adherirse a cada uno de los recipientes utilizados en un mismo caso clínico. De este modo el sistema de identificación es igual al sistema manual, la diferencia existe en la lectura de estas etiquetas que en vez de ser manual se realiza mediante aparatos electrónicos que leen automáticamente el identificador asociado. Actualmente existen en el mercado dos sistemas de identificación/trazabilidad electrónicos destinados a las TRAs, y aunque comparten la estrategia del marcaje del recipiente, existen algunas diferencias entre ellos:

- *Matcher*^{TM} de IMT International (Reino Unido), es un sistema basado en etiquetas autoadhesivas con código de barra y lectura mediante fotografía de

dicho código con un elemento de lectura transportable. En las etiquetas puede imprimirse también el nombre y otra información del paciente. Estas etiquetas son capaces de soportar el almacenaje en nitrógeno líquido y permanecer adheridas al recipiente después de la descongelación. Por otro lado, mediante este sistema es posible también trazar todos los productos utilizados para un caso clínico, leyendo el código de barras que lleva la etiqueta de fabricación de cada producto. En cualquier caso la lectura del código de barras requiere una correcta orientación de este.

- *RI Witness*^{TM} de Research Instruments (Reino Unido), es un sistema que utiliza tanto etiquetas autoadhesivas que incorporan un chip antena que emite una radiofrecuencia (RFID) como etiquetas autoadhesivas con código de barras de lectura mediante escáner laser. El primer tipo de etiquetas se utiliza para identificar los recipientes durante el transcurso del caso clínico, excepto para su criopreservación. Dado que las etiquetas de RFID sufren deterioro al someterse a temperaturas de -196^{o}C, *RI Witness*^{TM} recurre a los códigos de barras para identificar los soportes donde se almacenan las muestras criopreservadas. Una de las ventajas de las etiquetas de RFID es que no se requiere una orientación concreta para su lectura. No obstante, la lectura de este tipo de etiquetas esta limitada a una zona exclusiva de trabajo (plataforma de lectura situada en el interior de la campana de flujo, donde se realizan la mayoría de las manipulaciones de las muestras). El sistema de lectura por radiofrecuencia utiliza frecuencias de 13,56 Hz que han demostrado no afectar al potencial de desarrollo de embriones de ratón.

Estas herramientas suponen un gran avance gracias al desarrollo de robustos protocolos de supervisión que favorecen la correcta trazabilidad y ayudan indudablemente a preservarla. Sin embargo la infraestructura necesaria para implementar un laboratorio de FIV con estos sistemas requiere de una inversión económica elevada. Por otro lado, la metodología que utilizan para identificar las muestras reproductivas deja al descubierto puntos críticos en su trazabilidad. El hecho de que la etiqueta esté asociada al recipiente y que el marcaje se tenga que

realizar constantemente en todos los recipientes que se van utilizando en un caso, establece un riesgo de error humano repetido que hace peligrar la correcta trazabilidad de las muestras. El riesgo existe en cada momento de asociación de etiqueta a recipiente y en cada momento en el que la muestra ha de cambiar de recipiente. Además, centros que han testado estos sistemas de identificación/trazabilidad para muestras reproductivas han detectado errores de identificación sistemática (sin contemplar errores de asociación de etiqueta) en el 5,2% (*Matcher*^{TM}; Schnauffer et al., 2005) y el 1% (*RI Elemenents*^{TM}; Thornhill et al., 2013) de los eventos registrados.

1.1.3. Identificación y trazabilidad de muestras reproductivas obtenidas de animales de renta

La correcta identificación y trazabilidad de las muestras reproductivas durante las TRAs en animales de interés, a causa de su alto valor genético o por estar en peligro de extinción, es muy importante (Carolan et al., 1996; Ward et al., 2000; Bols 2005). Para obtener descendientes de animales de renta con valor genético, una de las opciones más rápida y eficiente es la producción de embriones a partir de la maduración y fecundación *in vitro* de ovocitos obtenidos mediante aspiración folicular transvaginal guiada por ultrasonidos (*Ovum Pick-Up*; OPU). Tras su cultivo *in vitro* estos embriones son transferidos a otras hembras receptoras sin valor genético encargadas de la gestación. Debido a que el número de COCs recolectados por hembra después de una sesión de OPU suele ser bajo (Petyim et al., 2003; Machado et al., 2006), la maduración y fecundación de los COCs y el cultivo de los embriones obtenidos de cada donante debe realizarse en pequeños grupos o individualmente (dependiendo del número de COCs obtenidos) para lograr preservar la información del pedigrí (Moessner et al. 1995). A pesar de que algunos grupos de investigación han conseguido tasas de blastocistos similares utilizando sistemas de cultivo colectivo e individual (Carolan et al., 1996; Vajta et al., 2000), este ultimo sistema está generalmente asociado a bajas tasas de

blastocistos y baja calidad de los embriones producidos (O'Doherty et al., 1997; Ward et al., 2000; Goovaerts et al., 2009). De hecho, las tasas más altas de formación de blastocistos y calidad embrionaria han sido logradas a través del cultivo colectivo o del co-cultivo con células somáticas (Moessner et al., 1995; Thibodeaux et al., 1995; Donnay et al., 1997;O'Doherty et al., 1997; Fujita et al., 2006; Reed, 2006). Se ha descrito que el cultivo en grupo de más de 20 embriones en volúmenes superiores a 20 μl y en concentraciones de un embrión por cada 1-10 μl de medio de cultivo ya sea en microgota o en pocillo proporciona tasas de blastocistos elevadas y reproducibles de blastocistos (Ferry et al., 1994; Donnay et al., 1997; O'Doherty et al., 1997; Fujita et al., 2006; Goovaerts et al., 2011). Los beneficios de estos sistemas de cultivo se atribuyen al intercambio de factores paracrinos entre embriones o entre células somáticas y embriones dentro del mismo medio de cultivo. Considerando estos beneficios, y como el número de COCs recuperados mediante OPU de una misma hembra es insuficiente para realizar un cultivo eficiente en grupo, se han desarrollado varios sistemas para cultivar embriones aislados pero compartiendo el mismo medio de cultivo. Estos sistemas se basan en la modificación del soporte de cultivo con el fin de otorgar un emplazamiento único a cada embrión dentro de un volumen de medio compartido:

- Sistemas de micropocillos (*Well-of–the-Well*, WOW): están basados en la modificación de la superficie del soporte de cultivo mediante la creación de micropocillos. Estos micropocillos pueden fabricarse como parte del mismo material que forma dicho soporte (Vajta et al. 2000) o mediante la adición de un dispositivo suplementario fabricado en polidimetilsiloxano (PDMS) que presenta el patrón de micropocillos (Akagi et al., 2010; Sugimura et al., 2010). Cada embrión se cultiva en un micropocillo, que comparte el medio de cultivo con el resto de micropocillos del soporte
- Sistemas de redes de poliéster (Booth et al., 2007; Somfai et al., 2010): están basados en la adición de redes de poliéster al soporte de cultivo convencional (microgota o pocillo). Estas redes están formadas por monofilamentos de

poliéster que al entrecruzarse forman poros de dimensiones determinadas, donde se emplazan los embriones aisladamente.

- Sistemas de superficies adherentes (Gopichandran 2006): están basados en la modificación de la superficie del soporte de cultivo convencional para convertirla en una superficie súper adherente. Principalmente se utiliza un producto comercial para conseguir esta modificación, el *Cell-Tack* (VWR International, Reino Unido). Se trata de un adhesivo basado en una formulación de proteínas polifenólicas extraídas del mejillón marino (*Mytilus edulis*), que son un componente clave de la cola que secreta para anclarse a estructuras sólidas en su entorno natural. Al colocar este adhesivo en la superficie del recipiente de cultivo, los embriones pueden adherirse a ella, y mantener una posición fija e identificativa durante su cultivo.

Todos estos sistemas otorgan un emplazamiento único a cada embrión, lo que permite identificar y trazar individualmente los embriones durante su cultivo colectivo *in vitro*. La posición que se otorga a cada embrión ha de ser mantenida durante todo el cultivo, permitiendo su identificación en todo momento. Así, si se requiere un cambio de medio, los embriones han de ser manipulados individualmente para luego ser posicionados en el mismo emplazamiento dentro del nuevo recipiente que contenga el medio correspondiente, lo que genera puntos críticos a la hora de preservar la correcta trazabilidad de los embriones.

Por otro lado los sistemas WOW comerciales son caros, y aunque su fabricación manual es posible suele ser laboriosa (Matoba et al., 2010). Además, los soportes de cultivo con sistemas de WOW y de red de poliéster han de ser transportados con extrema cautela ya que es muy fácil que un pequeño movimiento cause la deslocalización de los embriones perdiendo así su identificación (Goovaerts et al., 2010; Somfai et al., 2010).

A pesar de estas limitaciones, se ha sugerido que el microambiente de cultivo generado al utilizar estos sistemas de confinamiento proporciona un buen soporte nutricional, favoreciendo la dilución de los metabolitos tóxicos y la acumulación de factores autocrinos (Gopichandran et al., 2006; Somfai et al., 2010; Sugimura et al.,

2010), que se traduce en mejores tasas de desarrollo embrionario (Vajta et al., 2000; Gopichandran et al., 2006). Sin embargo, otras investigaciones no observan diferencias (Hoelker et al., 2009; Somfai et al., 2010; Dai et al., 2012) o tienen peores resultados en la producción *in vitro* de embriones utilizando estas plataformas (Pereira et al., 2005; Matoba et al. 2010), posiblemente debido a que todos estos sistemas de cultivo impiden el libre movimiento de los embriones, interfiriendo en la transmisión de los factores paracrinos y en los cambios de gradiente que el embrión genera cuando se desplaza libremente (Swain et al., 2011).

Un sistema de identificación individual directo que evitara el confinamiento estático de los embriones y permitiera su libre movimiento sería de gran interés para trazar embriones producidos de animales de elevado valor genético utilizando un sistema de cultivo contrastadamente eficiente como el cultivo colectivo.

1.2. Micropartículas codificantes

Simples, precisos y de fácil lectura, los códigos de barras se han convertido en el método más usado para trazar cualquier elemento en el mundo macroscópico. Actualmente, la demanda de trazar elementos se ha extendido al mundo microscópico con el fin de detectar y monitorizar directamente muestras de pequeñas dimensiones, que pueden llegar a ser del orden de moléculas (detección y monitorización de células, detección y separación de poblaciones celulares, detección de interacciones moleculares, etc.). Esta necesidad emergente ha conducido a la exploración de nuevos métodos de fabricación de códigos a escala muy pequeña (micro y nanométricos).

El código de barras convencional se basa en una serie de líneas verticales y espacios (elementos codificantes) de diferentes grosores. Varias combinaciones de estos elementos codificantes representan diferentes caracteres incorporados en el código. Teóricamente, variando el grosor, el orden secuencial y el número total de

barras y espacios es posible generar un número ilimitado de códigos. Para lograr códigos capaces de etiquetar muestras biológicas de pequeñas dimensiones estos han de ser suficientemente pequeños como para poder asociarse a la muestra sin alterarla, y suficientemente grandes como para poder identificar claramente los elementos codificantes que constituyen el código. Actualmente existen dos métodos de fabricación de micropartículas (1-100 μm) con la capacidad de producir códigos (microcódigos) donde la distinción de sus elementos codificantes sea posible.

1.2.1. Micropartículas con elementos codificantes asociados

La creación de estos microcódigos se caracteriza por el uso de micropartículas como sujetos de carga para elementos codificantes como moléculas o nanopartículas con características identificables. Los materiales que se utilizan normalmente para la fabricación de este tipo de micropartículas son el silicio y el poliestireno, debido a sus elevadas resistencias químicas. La asociación de elementos codificantes a estas micropartículas pueden llevarse a cabo mediante dos métodos:

- Unión externa de elementos codificantes (Figura 4A): los elementos codificantes pueden ser directamente unidos a la superficie de micropartículas mediante su encapsulación en partículas coloidales recubiertas de polielectrolitos que promueven interacciones electroestáticas con la micropartícula (Grøndahl et al., 2000). Otra opción es la creación de enlaces covalentes entre los propios elementos codificantes y la micropartícula de forma controlada y dirigida (Qin et al., 2007; Li et al., 2010).

- Internalización de elementos codificantes (Figura 4B, $4C_1$ y $4C_2$): estos métodos se basan en la asociación de los elementos codificantes con el interior de la micropartícula. Básicamente existen dos métodos para lograr esta asociación, la penetración de los elementos codificantes a través de los poros de una micropartícula polimérica (Gao et al., 2003 y 2004; Sathe et al., 2006), y la generación de una micropartícula *de novo* con los elementos codificantes formando parte de ella (Fulta et al., 1997; Vaidya et al., 2007; Wang et al., 2013).

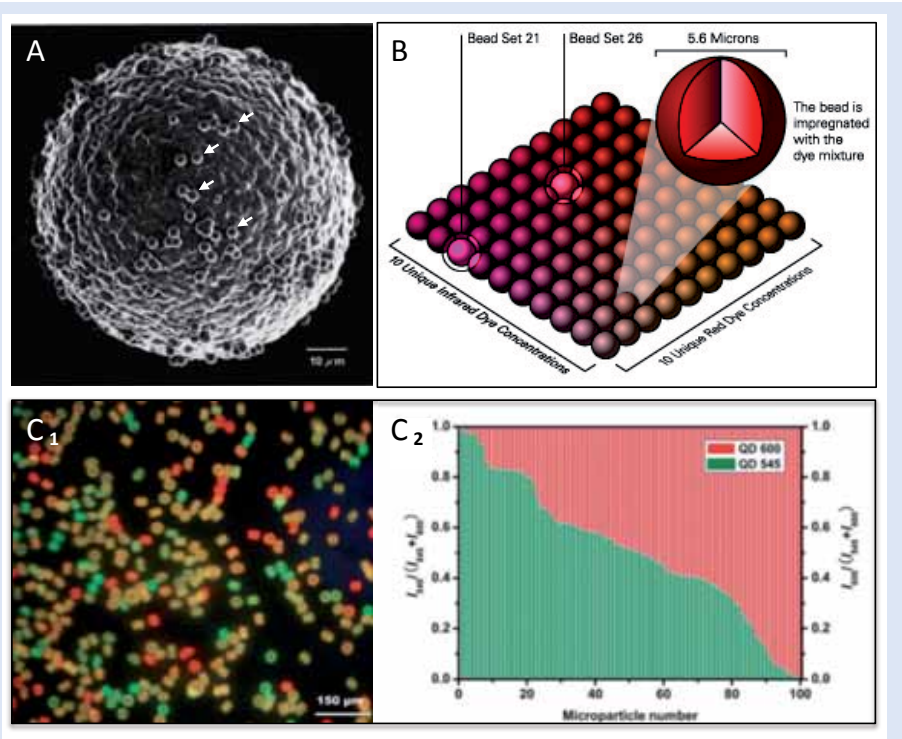

Figura 4. Micropartículas con elementos codificantes asociados. (A) Micropartícula codificada con múltiples partículas coloidales (flechas) adheridas a la superficie (Grøndahl et al., 2000). **(B)** Número de posibles variantes de microcódigos formados por micropartículas con diferentes proporciones de dos fluorocromos internalizados como elementos codificantes (MicroPlex Microspheres, Luminex, USA). **(C)** Micropartículas codificadas con *quantum dots* internalizados como elementos codificantes (C1). Variando la proporción de dos *quantum dots* se logran hasta 100 microcódigos diferentes (C2) (Ji et al., 2011).

Mientras que los métodos de fabricación de micropartículas portadoras de elementos codificantes por unión externa tienen una labilidad restringida, la

internalización proporciona a los elementos codificantes una protección frente a una posible degradación estructural propia y una estabilidad frente al deterioro causado por el medio. La internalización también individualiza los elementos codificantes previniendo su agregación, lo que pudiera alterar su señal y la correspondiente codificación que forman.

Indistintamente del proceso de fabricación, este tipo de microcódigos basa su capacidad de codificación en la incorporación de elementos codificantes que poseen propiedades ópticas o electrónicas únicas. Inicialmente, los elementos codificantes más utilizados para la fabricación de microcódigos fueron los fluorocromos orgánicos debido a su alta disponibilidad, perfil de emisión específico y buena sensibilidad. Como las barras y los espacios en un código de barras macroscópico, los perfiles de emisión de diferentes fluorocromos pueden combinarse en diferentes ratios para conseguir una codificación. La aplicabilidad de este tipo de codificación emergió con la comercialización de microesferas de poliestireno que por polimerización en suspensión incorporan dos fluorocromos orgánicos en diferentes proporciones pudiendo llegar a crear hasta 100 microcódigos diferentes (Figura 4B; Fulton et al., 1997; MicroPlex Microspheres, Luminex, USA).

Más recientemente han emergido unos nuevos elementos codificantes que mejoran las prestaciones otorgadas por los flurocromos orgánicos, las nanopartículas cuánticas o *quantum dots (QD;* Alivisatos 1996*)*. Mientras los fluorocormos orgánicos son altamente susceptibles al fotoblanqueado y a la inestabilidad química y poseen un espectro de emisión asimétrico de 30 a 50 nm de amplitud, los *QD* son muy resistentes al fotoblanqueado, presentan una elevada estabilidad química y sus espectros de emisión siguen una distribución Gaussiana simétrica de menor amplitud (25-40 nm), que varía en función de la medida y del material por el cual estén formados (Nirmal et al., 1999). Otra de las ventajas de los QD es que, independientemente del tipo, todos ellos exhiben un espectro de absorción con un pico común situado en la banda del ultravioleta (UV), lo que facilita significativamente el proceso de excitación requerido para la

lectura de los microcódigos formados por diferentes elementos codificantes fluorescentes (Vaidya et al., 2007). En función de combinaciones de emisiones (basadas en el tamaño del QD), de la intensidad de estas emisiones (basadas en el número de QD por micropartícula codificada) y del tamaño de la micropartícula huésped se pueden llegar a crear del orden de 10.000 códigos diferentes (Figura 4C; Wang et al., 2013).

La lectura de los tipos de microcódigos descritos en este apartado, al estar basados en la identificación de elementos codificantes fluorescentes, requiere la utilización de excitaciones lumínicas. Esto implica que los aparatos de lectura han de ser de una elevada sofisticación y poseer varias líneas de excitación (en el caso del uso de fluorocromos orgánicos como elementos codificantes). A demás el hecho de que la lectura de este tipo de micropartículas codificadas requiera de la excitación lumínica mediante luz ultravioleta hace que su uso para la identificación de células vivas no sea recomendable si se pretende mantener la viabilidad de estas, ya que como es conocido la exposición a luz ultravioleta produce citotoxicidad (McCarthy et al., 2006).

1.2.2. Micropartículas propiamente codificadas

Estos microcódigos basan su codificación en elementos gráficos creados en el momento de la fabricación de la micropartícula. En función del tipo de material y método de fabricación que se utilice para su fabricación pueden clasificarse en:

- Micropartículas de hidrogel fabricadas por litografía (Figura 5 A1 y A2): la producción de estas micropartículas se basa en la polimerización controlada de precursores monoméricos (como acrilatos y metacrilatos), elementos económicos, biocompatibles y alguno de ellos biodegradable (Hwang et al., 2009). Las micropartículas se fabrican por litografía, lo que implica la transferencia de un patrón que contenga las características geométricas (forma y dimensiones) de la partícula a formar al precursor del hidrogel, seguida de la polimerización de este. Actualmente existen tres métodos para

lograrlo, litografía por impresión (Glangchai et al 2008), fotolitografía (Kaehr et al., 2008) y litografía fluida (Dendukuri et al., 2007). Mientras que la primera se basa en la utilización de un molde negativo blando para la producción de las micropartículas, las otras dos utilizan mascaras que dejan pasar la luz (activador de la polimerización) siguiendo un patrón predefinido que es el que otorga las características geométricas a la partícula producida. Todas ellas utilizan la luz u otro tipo de radiación para inducir la polimerización y formación de las micropartículas. Contrastando con los métodos tradicionales de síntesis de micropartículas coloidales, que forman esferoides, este sistema permite crear formas y medidas muy variadas con una elevada precisión (Dendukuri et al., 2006). La misma forma puede ser utilizada como elemento codificante, aunque normalmente se utilizan elementos codificantes fluorescentes de forma integrada (Zhang et al., 2009) o por funcionalización de la superficie (Lee et al., 2010) para crear el patrón de codificación de estas micropartículas.

- Micropartículas formadas por materiales con índices de refracción distintos (Figura 5 B): un ejemplo es el uso de las propiedades ópticas de metales nobles para codificar las partículas (Nicewarner-Peña et al., 2001; Keating et al., 2003). Estos microcódigos metálicos se preparan por deposición electroquímica de distintos iones de metal (Ag, Pt, o Au) en una membrana porosa utilizada como molde/plantilla (Reiss et al., 2002; Walton et al., 2002), o mediante crecimiento catalítico asistido por láser de estos materiales (Gudiksen et al., 2002). La densidad electrónica de cada metal utilizado determina su refracción lumínica a una longitud de onda particular. Por ejemplo, a 430 nm el oro refleja la mitad de luz que la plata y, como resultado, bastoncillos formados por estos materiales forman códigos de barras que observados a esta longitud de onda presentan zona brillantes (plata) y oscuras (oro). Diferentes patrones de barras logrados con 2 metales y 13 segmentos pueden proporcionar 4160 distintas codificaciones (Nicewarner-Peña et al., 2001). El problema de estos microcódigos es que son

bastante frágiles, lo que impide una preservación estable de su codificación, característica indispensable que ha de poseer un elemento identificativo.

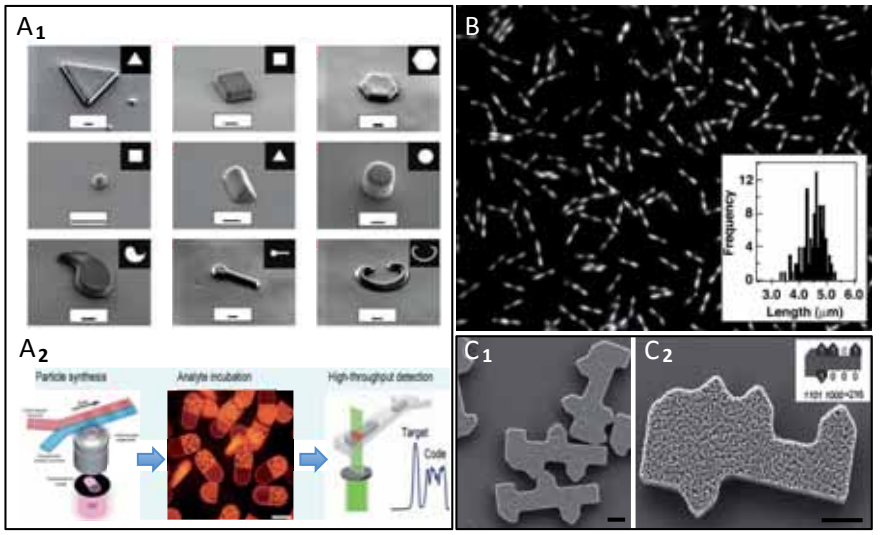

Figura 5. Micropartículas propiamente codificadas. (A) Micropartículas codificadas de hidrogel fabricadas por litografía (A1; Dendukuri et al., 2006) y fotolitografia (A2; Pregibon et al., 2007). **(B)** Micropartículas codificadas formadas por materiales (oro y plata) con distinto índice de refracción (Nicewarner-Peña et al., 2001). **(C)** Micropartículas fabricadas de materiales semiconductores (polisilicio) por tecnología de microsistemas (C1) con codificación binaria gráfica (C2) (Fernández-Rosas et al., 2009).

- Micropartículas de materiales semiconductores fabricadas por tecnología de microsistemas (Figura 5 C1 y C2): esta tecnología de fabricación permite desarrollar estructuras tridimensionales con formas complejas a micro y nanoescala (Collard et al., 2008). Estas micropartículas de pequeño tamaño, volumen y peso, pueden fabricarse por lotes en grandes cantidades con un coeficiente de variación casi inexistente, a bajo coste, en diferentes materiales y con diferentes técnicas. El silicio y el polisilicio (silicio en su forma policristalina) son los materiales más utilizados para fabricar estas micropartículas debido a sus propiedades físicas, químicas y térmicas (Petersen, 1982). Su fabricación se basa en las técnicas habituales de fabricación de circuitos microelectrónicos en combinación con técnicas

específicas para la estructuración tridimensional (Figura 6). Se suele partir de una fina lámina de material semiconductor llamada oblea, habitualmente de silicio, sobre la cual se depositan materiales como polisilicio, compuestos de silicio (Si_3N_4, SiO_2, SiC), metales o compuestos metálicos (Au, Ti, Ni, Al, ZnO), cerámicas y/o materiales orgánicos (polímeros, enzimas, DNA, anticuerpos). Para la fabricación de microdispositivos basados en silicio se utilizan tecnologías como la deposición física (*Physical Vapor Deposition*) o química (*Chemical Vapor Deposition*) de materiales, la fotolitografía para crear un patrón en estas capas, y el grabado para producir las formas necesarias (Madou, 2002). La estructuración de las capas mediante procesos fotolitográficos es la gran ventaja de esta tecnología. La fotolitografía se basa en la transferencia de un patrón a un material fotosensible por exposición selectiva a una fuente de radiación, como por ejemplo la luz UV. El patrón de la irradiación es transferido al material expuesto, lo cual modifica las propiedades entre las regiones expuestas y no expuestas. La eliminación de la resina que ha sido iluminada (resina positiva) o bien de la que no lo ha sido (resina negativa) proporciona una mascara para el material que esta debajo. La eliminación de las capas se puede realizar mediante grabado húmedo o seco. El grabado húmedo consiste en la disolución del material cuando es sumergido en una solución química, mientras que en el grabado en seco el material se pulveriza o disuelve mediante vapores de iones reactivos. Debido a su mejor rendimiento, se utilizan más las técnicas de grabado en seco: grabado por iones reactivos (*Reactive Ion Etching*, RIE) o grabado profundo por iones reactivos (*Deep RIE*). Con el fin de crear un elemento identificativo, las micropartículas fabricadas utilizando esta metodología basan su codificación en patrones gráficos distinguibles. Son microcódigos de pequeñas dimensiones (5-10 μm) de codificación binaria que permiten hasta 256 posibles codificaciones únicas, que pueden ser incrementadas al incrementar el número de bits que los forman (Fernández-Rosas et al., 2009; Gómez-Martínez et al., 2010). Como máxima distinción en referencia a todos

los métodos de fabricación de microcódigos descritos con anterioridad, no es necesario el uso de excitación lumínica para la lectura de la codificación los microcódigos fabricados por tecnología de microsistemas. Esto provee a este tipo de microcódigos la capacidad de ser leídos bajo microscopia óptica de luz transmitida, sin causar daño alguno al material biológico etiquetado.

El conjunto de estas características hacen que estas últimas micropartículas codificadas sean las más útiles para poder identificar muestras biológicas de medidas microscópicas como células eucariotas (Fernández-Rosas et al., 2009, 2011).

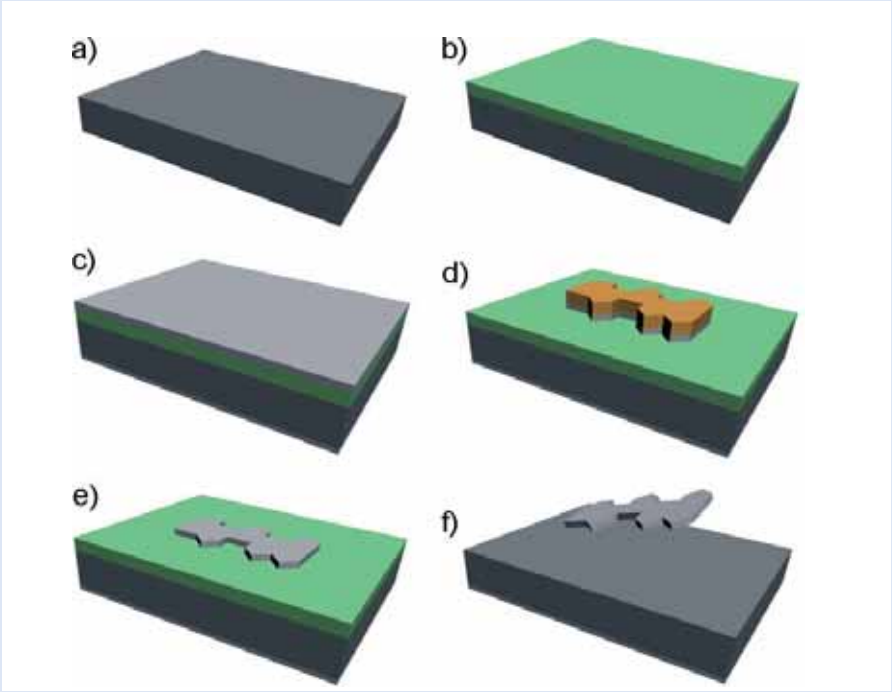

Figura 6. Fabricación de microcódigos de polisilicio mediante tecnología de microsistemas. (A) Oblea de silicio (en gris oscuro) como substrato inicial. **(B)** Deposición de una capa de óxido de silicio (en verde) como capa sacrificial y **(C)** una capa de polisilicio (en gris claro) como capa estructural. **(D)** Proceso fotolitográfico para definir las dimensiones laterales de los microcódigos. **(E)** Eliminación de la fotoresina restante (en naranja) y **(F)** liberación de los microcódigos mediante el ataque sacrificial de la capa de oxido de silicio (Fernández-Rosas et al., 2009).

2. OBJETIVOS

Esta tesis se enmarca dentro de un proyecto de investigación más amplio que tiene como objetivo la fabricación y caracterización de micro y nanoherramientas para el estudio de células vivas. Tanto en el proyecto de investigación como en esta tesis han participado activamente investigadores de distintos ámbitos científicos. Así, investigadores del Centro Nacional de Microelectrónica del CSIC han desarrollado y fabricado los microcódigos utilizados, investigadores de la Facultat de Farmacia de la Universitat de Barcelona han desarrollado un sistema de biofuncionalización para estos microcódigos y nuestro grupo, miembros de la Unitat de Biologia Cel·lular de la Universitat Autònoma de Barcelona, ha diseñado y llevado a cabo todos los experimentos descritos en esta Tesis.

El objetivo principal de esta tesis ha sido diseñar, desarrollar y aplicar un sistema de etiquetaje e identificación directo para muestras reproductivas, específicamente ovocitos y embriones bovinos y humanos, mediante su asociación a microcódigos codificados. Estos microcódigos deberán viajar conjuntamente con el ovocito/embrión durante todos los procesos a los que estos puedan ser sometido durante un ciclo de reproducción asistida. El sistema de etiquetaje e identificación directo deberá permitir un control de la trazabilidad más seguro y un menor riesgo de error de identificación de las muestras reproductivas que los sistemas actualmente disponibles para esta finalidad.

Para asumir dicho objetivo principal, los objetivos concretos fueron los siguientes:

1) Seleccionar, a partir de una serie de distintos diseños de microcódigo, el más eficiente para etiquetar e identificar embriones de mamífero durante su cultivo y manipulación *in vitro*.
2) Desarrollar, usando el modelo del ratón, un sistema eficiente de vinculación de los microcódigos a ovocitos/embriones que permita la retención del microcódigo y la identificación de las muestras durante su cultivo y manipulación *in vitro*.
3) Determinar si la presencia de los microcódigos o el sistema de asociación a los ovocitos/embriones afecta a la viabilidad o al potencial de desarrollo de los mismos.

4) Aplicar el sistema de etiquetaje directo a ovocitos y embriones humanos para mantener identificadas las muestras durante su procesamiento en el laboratorio de fecundación *in vitro*.
5) Aplicar el sistema de etiquetaje directo a embriones bovinos para permitir su cultivo colectivo manteniendo su identificación individual.

3. RESULTADOS

Articulo I (Objetivos 1, 2 y 3):

"A novel embryo identification system by direct tagging of mouse embryos using silicon-based barcodes"
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human reproduction

ORIGINAL ARTICLE ***Embryology***

A novel embryo identification system by direct tagging of mouse embryos using silicon-based barcodes

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BACKGROUND: Measures to prevent assisted reproductive technologies (ART) mix-ups, such as labeling of all labware and double-witnessing protocols, are currently in place in fertility clinics worldwide. Technological solutions for electronic witnessing are also being developed. However, none of these solutions eliminate the risk of identification errors, because gametes and embryos must be transferred between containers several times during an ART cycle. Thus, the objective of this study was to provide a proof of concept for a direct embryo labeling system using silicon-based barcodes.

METHODS: Three different types of silicon-based barcodes (A, B and C) were designed and manufactured, and microinjected into the perivitelline space of mouse pronuclear embryos (one to four barcodes per embryo). Embryos were cultured *in vitro* until the blastocyst stage, and rates of embryo development, retention of the barcodes in the perivitelline space and embryo identification were assessed every 24 h. Release of the barcodes after embryo hatching was also determined. Finally, embryos microinjected with barcodes were frozen and thawed at the 2-cell stage to test the validity of the system after cryopreservation.

RESULTS: Barcodes present in the perivitelline space, independently of their type and number, did not affect embryo development rates. The majority of embryos (>90%) retained at least one of the microinjected barcodes in their perivitelline space up to the blastocyst stage. Increasing the number of barcodes per embryo resulted in a significant increase in embryo identification rates, but a significant decrease in the barcode release rates after embryo hatching. The highest rates of successful embryo identification (97%) were achieved with the microinjection of four type C barcodes, and were not affected by cryopreservation.

CONCLUSIONS: Our results demonstrate the feasibility of a direct embryo labeling system and constitute the starting point in the development of such systems.

Key words: assisted reproductive technologies / embryo labeling / IVF mix-ups / traceability / silicon microtechnologies

Introduction

The increasingly high number of patients undergoing assisted reproductive technologies (ART) treatments worldwide (Wright *et al.*, 2008; Nyboe Andersen *et al.*, 2009) prevents the performance of totally individualized clinical and laboratory procedures. The simultaneity of independent ART cycles is unavoidable and, because of their caseload, fertility clinics cannot allocate separate work, incubation or storage areas for each patient sample. As a result, sample identification and mismatching errors may occur. In fact, since the first known case of an ART mix-up in 1987 in Manhattan, USA (Liebler, 2002), the accidental use of incorrect gametes or embryos during ART procedures has been reported in several centers around the world (Spriggs, 2003; Bender, 2006). Many of these mix-ups were detected because couples gave birth to babies of different skin color from their own or because fertility clinics later informed patients of the mistake, but it is possible that other cases could be going unnoticed.

Even though the occurrence of ART mix-ups is rare, their consequences are devastating for both the couples and fertility centers involved, leading to complex paternity suits and legal actions against the clinics which may end up in economic compensations. Therefore,

mechanisms to prevent these unintended accidents are currently being sought. Critical points during the clinical and laboratory procedures, when mismatching of gametes and embryos is most likely to occur, have been indentified: collection of oocytes and sperm, fertilization of oocytes by mixing them with sperm (IVF) or by injecting them with sperm (ICSI), transferring gametes or embryos between tubes or dishes, freezing and thawing of gametes or embryos, and embryo transfer into a patient (Magli *et al*., 2008). Medical-scientific societies such as the European Society for Human Reproduction & Embryology (ESHRE) or the Federación Latinoamericana de Sociedades de Esterilidad y Fertilidad (FLASEF), and regulatory bodies such as the Human Fertilisation and Embryology Authority (HFEA) in the UK, propose/mandate in their guidelines and codes of practice the permanent labeling of all labware to identify the source of the biological material inside the tube or dish, and the application of witnessing protocols to double check the identification of samples and the patients or donors to whom they relate, at all aforementioned critical points of the clinical and laboratory procedures. These measures, when rigorously followed, certainly minimize the risk of sample mismatching due to human error, but they do not eliminate it completely and they increase the already high workload of embryologists and clinicians and the costs of ART procedures. In fact, the efficacy of double-witnessing as a safeguard in the context of ART laboratories has been questioned, as errors can still occur due to involuntary automaticity. In addition, because embryologists must be continuously interrupted from their tasks by the need to double witness for other embryologists, this system may even have a side effect of increasing risk by creating distractions and interruptions to an embryologist's own work (Brison *et al*., 2004; Mortimer and Mortimer, 2005).

Recently, technological solutions for electronic witnessing that allow automation of the process of recognition and verification of sample identity and matching have been developed as an alternative to manual double witnessing. They include barcodes (Matcher^{TM}, Fertility QMS Ltd, UK) and radio frequency identification (RFID) labels (IVF Witness^{TM}, Research Instruments, UK) that can be attached to all labware containing gametes or embryos from a particular patient and automatically detected by a scanner or RFID reader connected to a computer, reducing the need for human intervention. The use of these electronic systems, especially RFID technology, is rapidly extending to fertility clinics worldwide (Schnauffer *et al*., 2005; Glew *et al*., 2006) and, in the UK, it is supported by the HFEA to substitute some manual witnessing steps. However, because gametes and embryos must be transferred from one container to another several times during the course of an ART cycle, the possibility of misidentification errors still exists.

To further minimize this risk, a method of labeling the gametes or embryos directly could be devised, so that the label would travel with the biological material throughout the entire ART process, from collection to transfer back to the patient. The labels should be made of a biocompatible material and should be small enough not to compromise gamete fertilization and embryo developmental potential, but large enough to hold a sufficient amount of information for sample identification purposes that could be read under a standard inverted microscope. In this sense, silicon-based barcodes on the low micrometer size range could be useful as embryo identification tags, as they fulfill all the aforementioned requirements. Moreover, they have already been successfully used as intracellular tags for human macrophages in culture, demonstrating their utility for individual cell tracking without affecting cell viability (Fernández-Rosas *et al*., 2009).

The aim of the present work was to provide a proof of concept for such a direct oocyte/embryo labeling system, by tagging pronuclear mouse embryos with silicon-based barcodes and monitoring them during *in vitro* culture. Several types of barcodes were designed and tested, and embryo labeling was accomplished by means of their microinjection into the perivitelline space. Rates of development, embryo identification, retention of barcodes in the perivitelline space during culture and release of barcodes after blastocyst hatching were determined to demonstrate the validity of this labeling approach. Moreover, the effectiveness of the labeling system after embryo cryopreservation was also investigated.

Materials and Methods

Animal care and procedures used in this study were conducted according to protocols approved by the Ethics Committee on Animal and Human Research of the Universitat Autònoma de Barcelona and by the Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya.

Collection of mouse embryos

Eight- to 12-week-old female mice of the hybrid strain B6CBAF1 (C57BL/6J × CBA/J) were used as embryo donors. Females were induced to superovulate by intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (Intervet, Spain) followed 48 h later by a second injection of 5 IU of human chorionic gonadotrophin (hCG; Farma-Lepori, Spain), and mated with males of the same strain. One-cell embryos were collected from the oviducts 25 h after hCG administration, and incubated for 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 cumulus cells. Denuded embryos were then washed twice in fresh H-KSOM and embryos with two pronuclei and a good morphology were selected. Selected embryos were incubated in KSOM culture medium containing both essential and nonessential amino acids and 1 mg/ml of bovine serum albumin (EmbryoMax, Millipore, Spain) at 37°C in a 5% CO_2 atmosphere until their use.

Design and fabrication of silicon-based barcodes

Three different types of silicon-based barcodes (A, B and C) with a binary code numerical representation were designed, fabricated and tested in this study (Fig. 1). Type A are three-dimensional (3D) silicon barcodes with a cylindrical shape and divided by engraved zones, allowing a total of six alphanumeric digits (bits) and, therefore, 64 different combinations (numbers 0–63). They are 10 μm in length and 3 μm in diameter. Type B and type C are two-dimensional (2D) polysilicon barcodes based on a horizontal matrix representation defined by either pentagonal (type B) or rectangular (type C) bits. Both types of 2D barcodes are 10 μm in length and 6 μm in width and have a thickness of 1 μm. They allow a total of 8 bits and, therefore, of 256 different combinations (numbers 0–255). However, because type C barcodes can be designed with either square (subtype C1) or rectangular (subtype C2) bits, the different combinations offered by this type of barcode is doubled (512 different barcodes). To allow the reading of the data in its correct orientation, all the barcodes are asymmetric and contain a start marker. The binary data contained in the barcode design can be easily converted to a decimal number (Fig. 1).

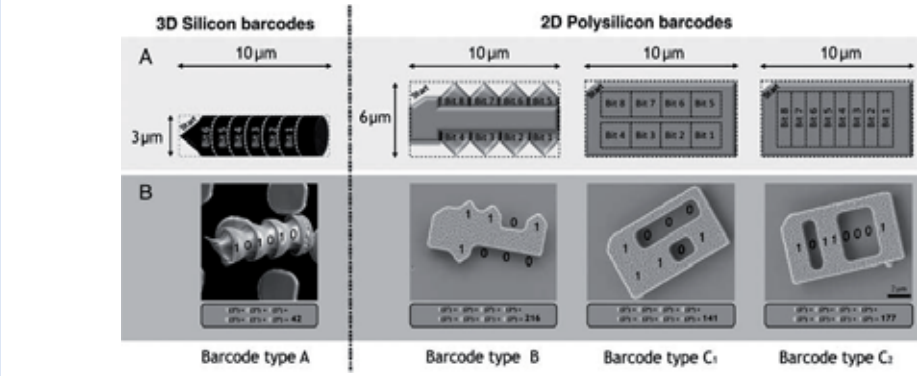

Figure 1 Design and dimensions of 3D (type A) and 2D (types B and C) silicon-based barcodes. (**A**) Schematic representation of the different types of barcodes used, showing shape, dimensions, number of bits and the start point. Note that type C1 and C2 barcodes only differ in the geometry of the bits. (**B**) Scanning electron microscope (SEM) images of some representative barcodes, in which the binary code number is indicated. The corresponding conversion of the binary code into a decimal number is detailed in the box below each image.

The three types of barcodes were fabricated on 4″ p-type (100) silicon wafers using silicon microtechnologies used for microelectromechanical systems (MEMS). The fabrication process for type B and C barcodes has been previously described (Fernández-Rosas *et al.*, 2009). Briefly, a plasma-enhanced chemical vapor deposition silicon oxide layer was deposited on the front side of the wafer to be used as a sacrificial layer for later release of the barcodes. Next, a 1 µm thick low-pressure chemical vapor deposition polysilicon layer (device layer) was deposited and the barcodes were patterned by a photolithographic step and a vertical polysilicon dry etching. The photoresist was removed by plasma etching, and the barcodes were released by the etching of the silicon oxide sacrificial layer in vapors of hydrofluoric acid.

Type A barcodes were fabricated using a similar process, but in this case a simple photolithographic step with 3 µm spot pattern on a previously grown silicon oxide layer, followed by sequential dry etching, was used to produce the cylindrical shape of the barcodes. Controlling vertical and non-vertical etch conditions allowed the definition of the binary code along the axis. The final fabrication step was a large non-vertical etching to release the barcodes (Gómez-Martínez *et al.*, 2009).

Microinjection of the barcodes into the perivitelline space

An Eppendorf TransferMan NK2 micromanipulator, a Burleigh Piezodrill and an Olympus IX71 inverted microscope were used to microinject the barcodes into the perivitelline space of the pronuclear stage embryos. Embryos were placed into a drop of H-KSOM medium in the micromanipulation dish and barcodes were transferred into a separate drop of 3% (w/v) polyvinilpirrolidone (Sigma, Spain) in H-KSOM, to avoid their precipitation and facilitate their aspiration with the injection micropipette. Several barcodes were first introduced into a blunt-ended microinjection pipette with an outer diameter of 10 µm. The pipette was then moved to the drop containing the embryos and used to drill a hole in the zona pellucida of an embryo with the help of a few piezo pulses. Next, the barcodes (1–4) were expelled into the perivitelline space of the embryo, as far away from the hole as possible to prevent their escape, and the pipette was gently withdrawn. Microinjection of the barcodes in 20 embryos took ~10 min.

Injected embryos were returned to the KSOM culture drops in the incubator and cultured until they hatched. Non-injected embryos were cultured in parallel as a control of development.

Embryo freezing and thawing

Embryos microinjected with barcodes and control non-injected embryos were frozen after 24 h of *in vitro* culture using a slow-freezing protocol (Costa-Borges *et al.*, 2009). Briefly, 2-cell embryos were first incubated for 7 min in H-KSOM containing 1.5 M propylene glycol (PROH; Fluka, Spain) at room temperature (RT). Embryos were then transferred to a drop of H-KSOM containing 1.5 M PROH and 0.1 M sucrose (Merck, Spain) and immediately loaded into 0.25 ml French-type straws (Minitube, Germany). Twelve to fifteen embryos were loaded per straw. The straws were thermo-sealed and placed in a controlled-rate freezer (Kryo 360, Planer, UK). Embryos were initially cooled at a rate of −2°C/min from RT to −7°C, the temperature at which manual seeding was performed. Next, they were cooled from −7 to −30°C at rate of −0.3°C/min, and then fast cooled to −150°C at a rate of −35°C/min (Lassalle *et al.*, 1985). Finally, the straws were directly plunged into liquid nitrogen at −196°C for storage.

At 1–7 days after cryopreservation, the straws were thawed by keeping them for 40 s at RT followed by 40 s at 30°C in a water bath. The embryos were then released from the straws and incubated for 15 min at RT in H-KSOM containing 0.3 M of sucrose. Finally, the embryos were incubated for 15 min in H-KSOM at 37°C and then transferred to KSOM culture medium and cultured at 37°C and in a 5% CO_2 atmosphere until they hatched.

Statistical analysis

All experiments were repeated at least three times on separate days and the results obtained in the replicated experiments were pooled. Data collected were analyzed by χ^2 test or Fisher's exact test. A probability value of $P < 0.05$ was considered to be statistically significant.

Experimental design

To test the validity of the proposed embryo labeling system and to select the most appropriate barcode design, a first set of experiments was performed in which a single barcode (type A, B or C) was microinjected into the perivitelline space of pronuclear stage embryos. Microinjected embryos were maintained in culture, together with a control group of non-injected embryos, until the blastocyst stage (96 h) and were monitored every 24 h under an inverted microscope to assess their developmental progression (development rate) and the presence of the microinjected barcode in the perivitelline space (retention rate). In addition, the number of developed embryos in which the barcode could be clearly read under the inverted microscope (200× magnification) was recorded (identification rate). It is important to point out that barcode reading was performed only by adjusting the focus on the microscope, without embryo manipulation. Therefore, only embryos with barcodes in the correct orientation could be successfully identified.

Once the most appropriate type of barcode was selected, a second set of experiments was performed in order to increase the identification rate. With this aim, two, three or four barcodes of the selected type were microinjected into the perivitelline space, and the microinjected embryos, together with a control group of non-injected embryos, were maintained in culture until the blastocyst stage (96 h). The same rates as in the previous experiments were determined every 24 h of culture (development, retention and identification rates) and, in this case, retention and identification rates were calculated considering only embryos that retained all the microinjected barcodes. In addition, in this set of experiments, blastocysts were kept in culture until they hatched and the number of hatched blastocysts that were totally free of the barcodes was determined (barcode release rate). Because some embryos were not able to complete hatching on their own, a short incubation with pronase (35 U/ml) was performed in these cases to help the zona pellucida digestion.

The last set of experiments was designed to test the validity of our embryo labeling system after an embryo freezing–thawing process. Pronuclear stage embryos were microinjected with the type and number of barcodes selected in the previous experiments and, after 24 h in culture, cleaved embryos that retained all the microinjected barcodes were cryopreserved. Two-cell embryos were thawed 1–7 days after freezing and maintained in culture until hatching. As in the previous set of experiments, the embryos were assessed every 24 h and the development, retention and identification rates, as well as the barcode release rate after hatching, were determined and compared with those obtained with the equivalent group of barcode-tagged embryos that were not cryopreserved.

Results

Selection of the optimal barcode design for embryo labeling

A total of 240 pronuclear-stage mouse embryos were microinjected, each with a single barcode (80 embryos per barcode type), and cultured in parallel to a group of 76 non-injected control embryos for 96 h. Development rates of barcode-tagged and control embryos were similar at all time points examined (Table I and Fig. 2), indicating that neither the microinjection process nor the presence of the polysilicon barcode in the perivitelline space affect embryo developmental potential.

Barcode retention rates during culture were higher than 90% in all three groups of tagged embryos, and no significant differences were detected among them (Table I). Retention rates did not differ significantly along the time points examined for each particular type of

Table I Rates of *in vitro* development and barcode retention in embryos microinjected with different types of barcodes.

Group	*n*	24 h		48 h		72 h		96 h	
		Development (%)	Retention* (%)	Development (%)	Retention* (%)	Development (%)	Retention* (%)	Development (%)	Retention* (%)
Control	76	65 (85.5)	–	65 (85.5)	–	65 (85.5)	–	64 (84.2)	–
Barcode A	80	70 (87.7)	70 (100)$^{\alpha}$	70 (87.7)	69 (98.6)$^{\alpha,\beta}$	70 (87.7)	66 (94.3)$^{\alpha,\beta}$	67 (83.8)	62 (92.5)$^{\beta}$
Barcode B	80	72 (90.0)	70 (97.2)	68 (85.0)	64 (94.1)	68 (85.0)	64 (94.1)	63 (78.8)	59 (93.7)
Barcode C	80	71 (88.8)	71 (100)	71 (88.8)	71 (100)	71 (88.8)	71 (100)	66 (82.5)	66 (100)

*Number of developed embryos that retain the microinjected barcode.
$^{\alpha,\beta}$Values with different superscripts within the same row differ significantly between time points ($P < 0.05$).

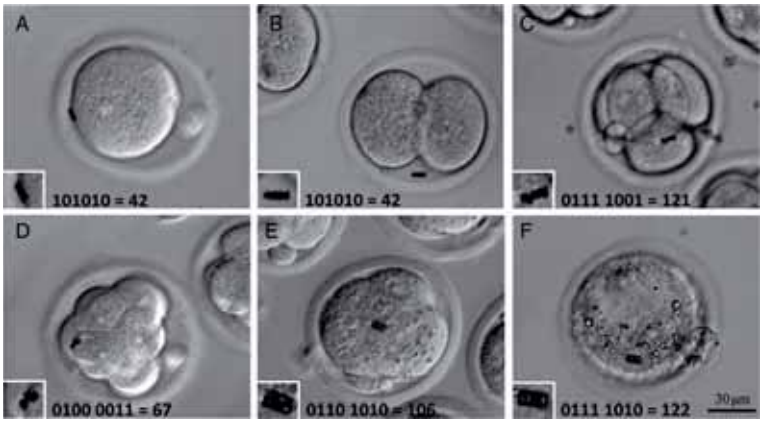

Figure 2 *In vitro* development of embryos microinjected with different types of polysilicon barcodes into their perivitelline space. (**A** and **B**) One- and 2-cell embryos labeled with type A barcodes. (**C** and **D**) Four-cell and compacting 8-cell embryos containing a type B barcode. (**E** and **F**) Morula and hatching blastocyst labeled with a type C barcode. Magnified images of the barcodes (insets) and their corresponding binary and decimal numbers are shown for each embryo.

barcode, except for a specific difference between 24 and 96 h for type A barcodes ($P = 0.026$). Therefore, the majority of microinjected barcodes, independently of their size and shape, remain in the perivitelline space from the pronuclear to the blastocyst stage.

Finally, with regard to embryo identification rates, values ranging from 30.5 to 58.6% were achieved and no significant differences were observed at any time point according to the type of barcode used (Table II). However, when the total number of identification processes performed during culture for each group of tagged embryos was considered, the rate of successful embryo identification was significantly higher when using type A (53.2%) than type B (41.2%) barcodes ($P = 0.008$), and the use of type C barcodes produced an intermediate result (48.0%). Comparison of identification rates along the time points examined only revealed significant differences for type B barcodes between 24 and 96 h ($P = 0.017$).

The results obtained in this first set of experiments indicated that none of the three types of barcodes tested was clearly superior to the others in terms of the parameters analyzed and, therefore, that all of them would be suitable for embryo tagging. In this context, we selected type C barcodes to proceed with the development of the embryo labeling system because this design allows for the highest number of combinations and it is the easiest to read under the inverted microscope.

Optimization of embryo identification rates

In a second set of experiments, aimed at increasing embryo identification rates, pronuclear stage embryos were microinjected with two, three or four type C barcodes into their perivitelline space (80 embryos per group) and cultured in parallel to a group of 49 non-injected control embryos for up to 120 h. Ideally, each embryo should have been injected with various copies of the same barcode, to simulate an eventual real situation in a clinical setting in which all embryos from the same patient or couple would be tagged with a unique barcode number. However, because type C barcodes were fabricated in all possible combinations in a single silicon wafer (including both subtypes C1 and C2) and they were mixed upon release, this was not possible at this stage of the research and the various barcodes injected into each embryo corresponded to different codes.

Rates of embryonic development up to the blastocyst stage (96 h) were similar among embryos microinjected with two, three or four barcodes and control non-injected embryos (Table III). When compared with embryos injected with a single type C barcode in the previous experiments (82.5% blastocyst rate, Table I), significant differences ($P = 0.022$) were only observed at 96 h for the group injected with four barcodes, which surprisingly showed a higher

Table II Identification rates of embryos microinjected with different types of barcodes.

Group	Identification (%)*				
	24 h	48 h	72 h	96 h	Total
Barcode A	41/70 (58.6)	35/69 (50.7)	38/66 (57.6)	28/62 (45.2)	142/267 (53.2)^{a}
Barcode B	37/70 (52.9)$^{\alpha}$	26/64 (37.5)$^{\alpha,\beta}$	27/64 (42.2)$^{\alpha,\beta}$	18/59 (30.5)$^{\beta}$	106/257 (41.2)^{b}
Barcode C	36/71 (50.7)	33/71 (46.5)	33/71 (46.5)	32/66 (48.5)	134/279 (48.0)^{a,b}

*Number of embryos that were successfully identified from those that developed and retained the microinjected barcode.
$^{\alpha,\beta}$Values with different superscripts within the same row differ significantly between time points ($P < 0.05$).
^{a,b}Values with different superscripts within the same column differ significantly between groups ($P < 0.05$).

Table III Rates of *in vitro* development and barcode retention in embryos microinjected with different numbers of type C barcodes.

Group	*n*	24 h		48 h		72 h		96 h	
		Development (%)	Retention* (%)	Development (%)	Retention* (%)	Development (%)	Retention* (%)	Development (%)	Retention* (%)
Control	49	45 (91.8)	–	45 (91.8)	–	45 (91.8)	–	44 (89.8)	–
Barcode Cx2	80	73 (91.3)	66 (90.4)	73 (91.3)	66 (90.4)	73 (91.3)	63 (86.3)	70 (87.5)	58 (87.5)
Barcode Cx3	80	70 (87.5)	63 (90.0)	70 (87.5)	59 (84.3)	70 (87.5)	58 (82.9)	68 (85.0)	57 (83.8)
Barcode Cx4	80	76 (95.0)	69 (90.8)	76 (95.0)	69 (90.8)	76 (95.0)	64 (84.2)	76 (95.0)	64 (84.2)

*Number of developed embryos that retain all of the microinjected barcodes.

percentage of embryos that reached the blastocyst stage (95.0%, Table III).

A high percentage of developed embryos retained all the microinjected barcodes during culture (82.9–90.8%; Table III) and no significant differences in retention rates were detected at any time point among the three groups of embryos microinjected with a different number of barcodes. Nor did retention rates differ along time in culture for any particular group. Compared with the 100% retention rate achieved in the previous experiments in the group of embryos injected with a single type C barcode, retention rates were significantly decreased at all time points when two to four type C barcodes were injected ($P = 0.0138$ to $P < 0.0001$). However, because the vast majority of embryos which did not retain all the microinjected barcodes only lost one of them, when the percentage of embryos retaining at least one of the microinjected barcodes was considered (98.5–98.7%, 98.5–100% and 100% for embryos injected with two, three or four barcodes, respectively), no significant differences were found at any time point when compared with the group of embryos microinjected with a single type C barcode.

In order to compare embryo identification rates when a different number of barcodes is present in the perivitelline space, only embryos that retained all the microinjected barcodes were initially considered (Table IV). Positive embryo identification required the successful reading of at least one of the barcodes present in the perivitelline space (Fig. 3). No significant differences were detected for any group along the different time points examined, but identification rates significantly differed between groups at all time points, specially between the groups having two or four barcodes ($P = 0.010$ to $P < 0.001$; Table IV). When compared with the injection of one type C barcode (Table II), injection of two or more barcodes resulted in a significant increase in identification rates at all time points examined ($P = 0.006$ to $P < 0.001$). As expected from these results, the total identification rate (considering the total number of identification processes performed during culture for each group of embryos) significantly differed between all groups ($P = 0.013$ to $P < 0.0001$), increasing from 48% for embryos injected with a single type C barcode (Table II) to 97% for embryos injected with four type C barcodes (Table IV).

In this set of experiments, embryos that reached the blastocyst stage by 96 h were maintained in culture for another 24 h to

Table IV Identification rates of embryos microinjected with different numbers of type C barcodes.

Group	Identification (%)*				
	24 h	48 h	72 h	96 h	Total
Barcode Cx2	50/66 (75.7)^{a}	49/66 (74.2)^{a}	45/63 (71.4)^{a}	46/58 (79.3)^{a}	190/253 (75.1)^{a}
Barcode Cx3	59/63 (93.6)^{b}	50/59 (84.7)^{a,b}	54/58 (93.1)^{b}	49/57 (86.0)^{a}	212/237 (89.4)^{b}
Barcode Cx4	65/69 (94.2)^{b}	67/69 (97.1)^{b}	61/64 (95.3)^{b}	64/64 (100)^{b}	257/265 (97.0)^{c}

*Number of embryos that were successfully identified from those that developed and retained all of the microinjected barcodes.

$^{a-c}$Values with different superscripts within the same column differ significantly between groups ($P < 0.05$).

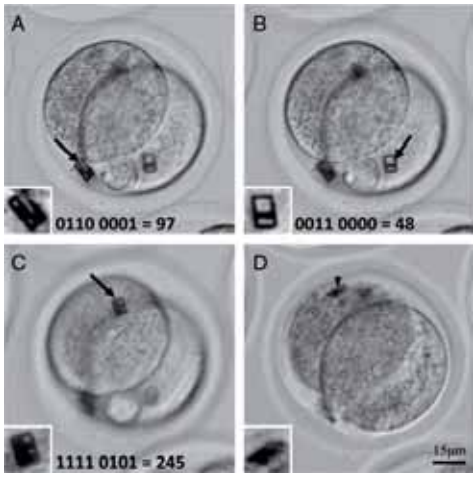

Figure 3 Identification process of a 2-cell embryo containing four type C barcodes in its perivitelline space. Identification was performed under an inverted microscope (200× magnification) by adjusting the focus without reorienting the embryo. (**A**–**C**) Images of various focal planes at which three of the barcodes could be clearly read (type C1 in A and C; type C2 in B). (**D**) The fourth barcode present in the perivitelline space could not be read, due to its incorrect orientation. Magnified images of the barcodes (insets) and, when readable, their corresponding binary code and decimal number are shown.

determine the fate of the barcodes after embryo hatching. In blastocysts derived from embryos injected with two barcodes, barcodes usually remained inside the empty zona pellucida or were released when embryos hatched, so that most of the blastocysts were totally free of the barcodes after hatching (44/58, 75.9% release rate). In the remaining blastocysts (14/58, 24.1%), at least one of the barcodes was firmly adhered to the embryo surface and could not be liberated even after rough pipetting of the hatched embryos (Fig. 4). Complete barcode release rates significantly decreased ($P = 0.026$ to $P < 0.001$) as the number of barcodes injected into the perivitelline space was increased: 54.4% (31/57) for embryos injected with three barcodes and 17.2% (11/64) for those injected with four barcodes.

Cryopreservation of the barcode-tagged embryos

In the last set of experiments, embryos microinjected with four type C barcodes at the pronuclear stage were cultured for 24 h and cryopreserved at the 2-cell stage ($n = 148$), in parallel to control non-injected embryos ($n = 49$). After thawing, embryos were cultured for 72 h, and no significant differences were found between the two groups in development rates (Table V). Development of frozen-thawed embryos carrying four barcodes in their perivitelline space was also similar at all time points examined to that of their non-cryopreserved counterparts from the previous set of experiments (Table III).

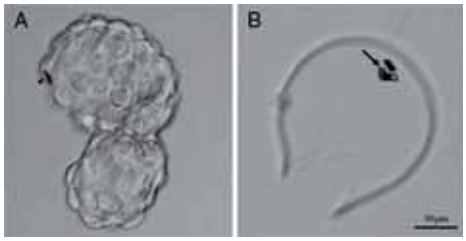

Figure 4 Adhesion of barcodes to the embryo surface. (**A**) Hatched blastocyst showing one type C barcode adhered to its surface (arrowhead). (**B**) In the corresponding empty zona pellucida, the three remaining barcodes are left.

The majority of cryopreserved embryos retained all four microinjected barcodes after thawing and culture (94.5–96.2%; Table V), and these retention rates were significantly higher than those obtained in the group of embryos microinjected with four type C barcodes that were not cryopreserved, at 48 h ($P = 0.014$) and 72 h ($P = 0.028$). However, because barcode loss mainly takes place during the 1-cell to 2-cell transition and because retention rates in cryopreserved embryos were calculated from the number of 2-cell embryos thawed, these differences can probably be attributed to the different starting point in the calculation of these rates for non-cryopreserved (1-cell) and cryopreserved (2-cell) embryos. In fact, in both groups of tagged embryos 100% of them retained at least one of the microinjected barcodes up to the blastocyst stage.

All the barcodes maintained their integrity after freezing and thawing. Identification rates of 90.5% (115/127), 93.4% (114/122) and 97.5% (118/121) were achieved after 24, 48 and 72 h of culture, respectively, of the frozen-thawed microinjected embryos. This means that out of the total number of identification processes performed during culture, in 93.8% (347/370) of the embryos at least one of the barcodes could be successfully read, a rate that was similar to that of the non-cryopreserved group (97%).

As in the previous set of experiments, the complete barcode release rate after embryo hatching (96 h of culture after thawing) was very low (16/121, 13.2%), demonstrating again that when four barcodes are present in the perivitelline space the probability that at least one of them remains adhered to the embryo surface after hatching is high.

Discussion

In this work, a first step towards the development of a safe and reliable direct oocyte/embryo identification system is presented. Once developed, such a system could minimize the risk of sample misidentification and mismatching errors during ART procedures and greatly reduce the steps in the clinical and laboratory processes at which manual double-witnessing should be performed.

This novel approach of direct sample labeling is based on the use of micrometer-sized silicon-based barcodes containing bits that are large enough ($\geq 1\ \mu m^2$) to be visible under a standard optical microscope.

Table V **Rates of *in vitro* development and barcode retention after thawing of embryos microinjected with four type C barcodes and cryopreserved at the 2-cell stage.**

Group	*n*	24 h		48 h		72 h	
		Development (%)	Retention* (%)	Development (%)	Retention* (%)	Development (%)	Retention* (%)
Control	49	43 (87.8)	–	41 (83.7)	–	41 (83.7)	–
Barcode Cx4	148	132 (89.2)	127 (96.2)	128 (86.5)	122 (95.3)	128 (86.5)	121 (94.5)

*Number of developed embryos that retain all of the microinjected barcodes.

Therefore, setting up such a system in an embryology laboratory would be straightforward as no special equipment would be needed, other than an inverted microscope equipped with a micromanipulator, a camera and a computer, which most embryology labs already have. Even though manual eye reading of the barcodes was performed in this initial work reported here, a computer software for the automatic reading of the barcodes is now being developed, which will facilitate a faster and more reliable reading.

As a proof of concept for this novel labeling system of oocytes and embryos, mouse embryos at the pronuclear stage were used as sample models and the barcodes were microinjected into the previtelline space. Because our laboratory routinely performs mouse nuclear transfer experiments, our microinjection setup is adapted to work with a piezodrill and this system was used for the experiments described in the present work. However, piezo-assisted microinjection is not a requirement for the microinjection of the barcodes, as long as beveled micropipettes are used. In fact, because the hole created in the zona pellucida with a blunt-ended piezo-driven pipette is much bigger than that created with a beveled pipette, the use of a conventional microinjection system with beveled pipettes for the microinjection of the barcodes may have resulted in a higher retention rate of the barcodes in the perivitelline space during embryo culture.

In the first part of our study, the validity of the approach was investigated and three different types of barcodes were tested to select the most appropriate for embryo labeling and identification. Barcodes microinjected into the pervitelline space of the embryos clearly had no effect on their *in vitro* developmental potential up to the blastocyst stage. These results were expected, as we had previously demonstrated that silicon and polysilicon microparticles internalized into human macrophages by phagocytosis do not affect cell viability and that no effect on *in vitro* development is seen when these microparticles are microinjected into the cytoplasm of mouse pronuclear embryos (Fernández-Rosas *et al.*, 2010). In addition, type B barcodes, the same as the ones used in the present work, had already been used successfully to label and track human macrophage cells in culture without any apparent effects on cell viability (Fernández-Rosas *et al.*, 2009).

Most of the injected barcodes remained in the previtelline space during embryo culture and development, and 40–50% could be successfully read, allowing the correct identification of the corresponding embryos. It is important to note that embryo identification was performed without manipulating the embryos, because it was our intention to simulate the eventual situation in which an automatic reading system would be used. Therefore, successful reading of the barcodes in these circumstances is totally dependent on barcode orientation. It is probable that if embryos had been manipulated to change their orientation, all of the barcodes could have been finally read and embryo identification rates of 100% could have been obtained. In fact, anticipating the importance of barcode orientation for successful reading, 3D type A barcodes were designed as we reasoned that they would be easier to read than 2D type B and C barcodes in any orientation. This did not turn out to be the case, as identification rates for embryos containing type A barcodes were similar to those of embryos containing type C barcodes, and only slightly higher than those of embryos containing type B barcodes.

As all the parameters tested in the first set of experiments were similar for the three types of barcodes tested, our selection had to be based on other barcode properties. In their current design, 2D barcodes (with 8 bits) allow a higher number of combinations than 3D barcodes (with only 6 bits). Moreover, the bits of type C barcodes can be designed with two different geometries, allowing twice the number of combinations than type B barcodes. In addition, we found that type C barcodes were easier to read, at least manually, than type B or even type A barcodes. For all these reasons, we considered type C barcodes as the most appropriate for our embryo labeling approach, as a higher number of embryos could be potentially labeled.

Next, we focused on increasing embryo identification rates to a value as close as 100% as possible. Because correct orientation of the barcode is key for its successful reading, the number of barcodes microinjected per embryo was increased in order to maximize the probability that at least one of them remains properly oriented for its reading. Increasing the number of barcodes present in the perivitelline space up to four had no detrimental effects on embryo development, and the percentage of embryos that retained at least one of the microinjected barcodes during all preimplantation development (and, therefore, could be potentially identified) was similar, independently of the number of barcodes injected, and very close or equal to 100%. Nonetheless, as expected, identification rates significantly increased in parallel to the number of barcodes present, reaching a maximum of 97% for embryos receiving four type C barcodes.

Having determined that tagging embryos with four type C barcodes is the optimal condition in terms of identification rates, the next step was to determine whether barcodes would be able to withstand cryopreservation and whether the effectiveness of the embryo labeling system would be maintained. Cryopreservation of surplus embryos is a routine procedure at fertility centers, and because it involves multiple transfers of embryos from one container to another during both freezing and thawing processes, it is considered as one of the critical

points when mismatching of embryos may occur. Our results clearly show that embryo freezing and thawing have no detrimental effects on the developmental potential of tagged embryos, barcode integrity and retention, or embryo identification rates. Therefore, the barcode-based embryo labeling approach presented here is also reliable after cryopreservation.

One of the unexpected hurdles of the system was the low release rate of barcodes observed after embryo hatching, especially when four barcodes per embryo were used. Because adherence of barcodes to the embryo surface was never observed in pre-blastocyst stages, two non-excluding explanations for this phenomenon are possible. First, the reduction or even disappearance of the perivitelline space when blastocysts expand may facilitate a close contact between the barcode and the trophectoderm cells surface, thus inducing their adhesion. Second, changes in cell surface that accompany the formation of trophectoderm cells (Yamanaka *et al.*, 2006) may promote the adhesion of the barcodes. Barcodes should accompany embryos throughout the entire *in vitro* procedures, so that they can be indentified and tracked at every critical step, but they should be released from the embryos before implantation. Even though the effect of barcodes adhered to the blastocyst surface on embryo implantation has not been assessed, this situation should be avoided if an embryo labeling system like the one described here is to be eventually applied in a clinical setting. It is possible that coating of the barcodes surface with a hydrophobic biocompatible compound could prevent their adhesion to the embryos. Alternatively, a biodegradable material could be used for the fabrication of the barcodes.

In summary, the results presented here demonstrate the feasibility of a direct embryo labeling system and constitute the starting point in the development of such systems. Even though pronuclear embryos were used in the experiments reported here, the same barcode-based labeling approach could also be applied to embryos at later developmental stages and to oocytes. In fact, in a clinical setting, labeling of samples at an early point of the ART procedure would be the best approach to minimize the risk of sample mismatching, and microinjection of the barcodes could be performed in oocytes at the time of ICSI. In the case of embryos undergoing preimplantation genetic diagnosis, in which a relatively large opening is created in the zona pellucida during blastomere biopsy, a higher number of barcodes might be injected into the perivitelline space to reduce the possibility of losing all the barcodes.

Compared with current technological solutions for sample identification and tracking during ART procedures, a direct sample labeling approach would minimize even further the risks of human errors in sample identification and mismatching because the labels would travel together with the samples throughout all the steps in the process, even when samples are moved from one container to another. However, because the approach is not applicable to sperm cells, mismatching of sperm and oocytes at the fertilization step could not be avoided with this system. In a barcode-based system like the one described here, sample identification takes place under a standard inverted microscope, avoiding exposure of oocytes and embryos to potentially harmful radio waves or lasers and the need for expensive specialized equipment.

In spite of the promising results obtained so far, the approach reported here for direct embryo labeling has some limitations, such as barcode adhesion to the embryo surface after hatching or the need for micromanipulation to label each individual embryo. Current work in our laboratory is focused on overcoming these limitations, and alternative methods of barcode incorporation into oocytes/embryos are being pursued. In particular, modification of the barcode surface aimed at the selective attachment of barcodes to the outer surface of the zona pellucida by either physical or chemical means is being investigated.

Authors' roles

L.B., C.N., J.S. and E.I. conceived the project and designed the experiments; J.E. and J.A.P. designed the barcodes; R.G.M. and M.D. fabricated 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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Articulo II (Objetivo 2):

"Biomolecule screening for efficient attachment of biofunctionalized microparticles to the zona pellucida of mammalian oocytes and embryos"

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Biomolecule screening for efficient attachment of biofunctionalized microparticles to the zona pellucida of mammalian oocytes and embryos

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Abstract Individual tagging of oocytes and embryos through the attachment of micrometer-sized polysilicon barcodes to their zona pellucida (ZP) is a promising approach to ensure their correct identification and traceability in human assisted reproduction and in animal production programs. To provide barcodes with the capacity of binding to the ZP, they must be first biofunctionalized with a biomolecule capable of binding to the ZP of both oocytes and embryos. The aim of this work was to select, among an anti-ZP2 antibody and the two lectins wheat germ agglutinin (WGA) and phytohemagglutinin-L, the most optimal biomolecule for the eventual biofunctionalization of barcodes, using mouse oocytes and embryos and commercially available microspheres as a model. Despite the anti-ZP2 antibody showed the highest number of binding sites onto the ZP surface, as determined by field emission scanning electron microscopy, the binding of anti-ZP2-biofunctionalized microspheres to the ZP of cultured oocytes and embryos was less robust and less stable than the binding of lectin-biofunctionalized ones. WGA proved to be, among the three candidates tested, the most appropriate biomolecule to biofunctionalize microparticles with the aim to attach them to the ZP of both oocytes and embryos and to maintain them attached through oocyte activation (zona reaction) and *in vitro* culture up to the blastocyst stage. As saccharides recognized by WGA are highly abundant in the ZP of most mammalian species, WGA-biofuncionalized microparticles would be able to attach to the ZP of oocytes/embryos of species other than the mouse, such as humans and farm animals.

Keywords Wheat germ agglutinin · Phytohemagglutinin-L · Anti-ZP2 antibody · Oocyte tagging · Embryo tagging

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1 Introduction

The correct identification and traceability of gametes and embryos is a must in human assisted reproduction and in animal production programs to avoid mismatching errors. Because of the limitations of the current systems (individual culture, labeling of labware, double- or electronic-witnessing protocols, etc.), our group is working on the development of a direct oocyte/embryo tagging system which allows their unequivocal identification. Our first approach was based on the microinjection of encoded polysilicon microparticles (barcodes) into the perivitelline space of pronuclear (PN) embryos, using the mouse as a model (Novo et al. 2011). Despite the excellent results in terms of embryo viability and identification rates obtained with this approach, we noticed that a significant number of barcodes remained attached to the embryo surface after hatching. As, both in a human and in an animal setting, tagged embryos would have to be transferred at some point to a patient or a receptor female, respectively, it is important that embryos are freed of the barcodes after hatching so that implantation is not affected by the possible presence of a barcode. This unexpected hurdle led us to devise an alternative tagging approach in order to avoid the contact between the barcode and the embryo. This new approach consists in the attachment of the barcodes to the outer surface of the zona pellucida (ZP) by chemical means, i.e. by biofunctionalization of the barcode surface with a biomolecule capable of binding to the ZP. As a first step towards the

development of this new tagging system, the appropriate biomolecule to ensure a robust and stable binding of the barcodes to the ZP of both oocytes and embryos must be selected.

The ZP is an extracellular coat that surrounds mammalian oocytes and preimplantation embryos up to the hatching stage. It is composed of highly glycosylated proteins, known as ZP1, ZP2, ZP3 and, in some species, ZP4 (Goudet et al. 2008). The biochemical composition of the ZP changes after fertilization due to the action of the glycosidases and proteases released by the cortical granules, a process known as zona reaction (Wassarman 1987a, b). The main biochemical modifications associated with the zona reaction are the modifications of ZP2 and ZP3 proteins (spermatozoa secondary and primary ligand, respectively) and the changes in the ZP saccharide moiety, which altogether result in a permanent block against polyspermy (Rankin and Dean 2000). Biomolecules such as sperm-egg binding proteins, antibodies, and lectins have all proved a ZP binding capacity (Wassarman 1987a, b). But whereas sperm-egg binding proteins cannot bind to the ZP after fertilization (Benoff 1997), antibodies and lectins can (Avilés et al. 1997) and seem therefore more feasible candidates to provide barcodes with the capacity of binding to the ZP of both oocytes and embryos.

The rat monoclonal antibody against mouse ZP2 (anti-ZP2), clone IE-3, is a 150 kDa IgG that has shown high and specific affinity against the amino acids 114–129 of mouse ZP2 glycoprotein, the major component of the ZP (East and Dean 1984). On the other hand, the wheat germ agglutinin (WGA) from the cereal *Triticum vulgaris* is a dimmer with a molecular mass of 36 kDa which belongs to the chitin-binding lectins family (Nagata and Bruger 1974) and shows its highest affinity to N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid (sialic acid) monosaccharides (Bhavanandan and Katlic 1979). Finally, phytohemagglutinin-L (PHA-L) is a tetrameric leucoagglutinating lectin with a molecular mass of 126 kDa (Sharon and Lis 2004) from the seed of the legume *Phaseolus vulgaris* which belongs to the L-type lectins family (Etzler 2009). Although PHA-L carbohydrate-binding activity can be inhibited by monosaccharides (Sharon and Lis 2004), the minimal structural unit required for high-affinity binding is the pentasaccharide Galβ1→4GlcNAcβ1→2[Galβ1-4GlcNAcβ1-6]Man (Hamelryck et al. 1995). The saccharides recognized by WGA and PHA-L are abundant in the mouse ZP and it has been shown that both lectins can bind to the mouse ZP before and after fertilization (Avilés et al. 1997; Jiménez-Movilla et al. 2004).

In this context, the main goal of the present work was to select the most optimal biomolecule, among an anti-ZP2 antibody and the two lectins WGA and PHA-L, to eventually biofunctionalize barcodes to be attached to the ZP of both oocytes and embryos. To this aim, mouse oocytes and embryos, and commercially available microspheres, which can be more easily and efficiently biofunctionalized than polysilicon barcodes, were used as a model system. The biomolecule selection was carried out taking into account four different parameters: the location of binding sites for each biomolecule in the ZP before (oocytes) and after fertilization (PN embryos); the number of binding sites per area of ZP surface for each biomolecule in both oocytes and embryos; the potential toxicity of each biomolecule (either free or attached to the microspheres) during *in vitro* embryo culture; and the capacity of biofunctionalized microspheres to attach to the ZP of both oocytes and embryos and to remain attached after the zona reaction and during *in vitro* embryo culture up to the blastocyst stage.

2 Materials and methods

Animal care and procedures used in this study were conducted according to protocols approved by the Ethics Committee on Animal and Human Research of the Universitat Autònoma de Barcelona and by the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

2.1 Collection of oocytes and pronuclear embryos

Hybrid B6CBAF1 (C57BL/6 J×CBA/J) female mice (age, 8–12 weeks) were used as oocyte and embryo donors. Females were induced to superovulate by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Intervet, Spain) followed 48 h later by a second injection of 5 IU of human chorionic gonadotropin (hCG; Farma-Lepori, Spain). To obtain embryos, females were mated with males of the same strain immediately after hCG injection. Oocytes and PN embryos were collected from the oviducts 14 h and 25 h after hCG administration, respectively. Then, they were incubated for 5–10 min at 37 °C in Hepes-buffered potassium simplex optimized medium (H-KSOM) supplemented with 300 U/ml (oocytes) or 150 U/ml (embryos) of hyaluronidase (Sigma, Spain) for dispersion of cumulus cells. Denuded oocytes and PN embryos were then washed twice in fresh H-KSOM.

2.2 Detection of the binding of biomolecules to the zona pellucida

Oocytes and PN embryos were fixed for 20 min with 4 % paraformaldehyde in KSOM-H at room temperature (RT) and rinsed three times in 0.01 M phosphate-buffered saline (PBS, pH 7.4). To detect the binding capacity of the two lectins to the ZP, fixed oocytes and embryos were incubated for 30 min with 10 μg/ml of Alexa Fluor 594-conjugated WGA (W11262, Invitrogen, USA) or PHA-L (L32456, Invitrogen) in PBS at 37 °C in the dark. To detect the binding capacity of the anti-

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ZP2 antibody, fixed oocytes/embryos were incubated for 1 h at RT with 4 μg/ml of rat monoclonal primary antibody (sc32752, Santa Cruz Biotechnology, USA) in PBS containing 0.3 % bovine serum albumin (PBS-BSA). After three rinses in PBS-BSA, specimens were next incubated for 30 min with 0.02 μg/ml of Alexa Fluor 594-conjugated rabbit anti-rat IgG secondary antibody (A21211, Invitrogen) at 37 °C in the dark. Finally, the specimens were washed three times in PBS-BSA and mounted onto slides in PBS containing 25 mg/mL of sodium azide.

Samples were analyzed under an Olympus FluoView FV1000 confocal laser scanning microscope (CLSM; Olympus, Germany) and equatorial XY scans allowed to observe the labeling distribution of each biomolecule throughout the ZP of oocytes and PN embryos.

2.3 Quantification of biomolecules binding sites onto the zona pellucida surface

Oocytes and PN embryos were processed following an adaptation of the protocol described by De Harven et al. (1984). Briefly, the specimens were fixed with 4 % paraformaldehyde in KSOM-H for 20 min at RT and rinsed three times with PBS-BSA. For lectin binding quantification, specimens were incubated with 39.5 nM of 20 nm colloidal gold-conjugated WGA (GP2101, EY Laboratories, USA) or PHA-L (GP1801, EY Laboratories) in PBS-BSA during 30 min at 37 °C. For antibody binding quantification, specimens were incubated during 1 h at RT with 39.5 nM of primary anti-ZP2 antibody in PBS-BSA, rinsed three times in PBS-BSA, and then incubated for 30 min at 37 °C with 59.2 nM of 20 nm colloidal gold-conjugated goat anti-rat IgG antibody (GAF-461, EY Laboratories). After incubation, all specimens were rinsed thoroughly with 0.1 M sodium cacodylate buffer (SCB, pH 7.4), fixed with 2.5 % glutaraldehyde in 0.1 M SCB for 2 h at RT and post-fixed with 1 % osmium tetroxide in 0.1 M SCB during 2 h at RT. Following dehydration in a graded ethanol series (30 % to 100 %), samples were critical point-dried using CO_2 (CPD 030 critical point dryer, Bal-Tec, Germany) and mounted onto aluminum stubs covered with an auto-adhesive carbon film. The samples were examined at 1.2 kV, 100 pA and 3 mm of working distance under a field emission scanning electron microscope (FESEM, Merlin Carl Zeiss AG, Germany) using secondary electron (SE) and backscattered electron in-lens (EsB) detectors. Thus, at a magnification of ×80 000, images of three random areas of the ZP were captured for each specimen, first using the SE detector to establish the region of interest for the analysis (Fig. 1 (a_1)), and second using the EsB detector to determine the distribution of the colloidal gold nanoparticles on the ZP surface (Fig. 1 (b_1)). The EsB grid was established at 800 V, which allowed enough contrast to differentiate nanoparticles from the ZP.

As the ZP is a porous structure, to quantify the number of binding sites per surface area the region of interest was defined without considering the pores. Consequently, holes in the SE detector image, corresponding to the pores, were extracted (Fig. 1 (a_2)) and the area of the region of interest was measured from the SE detector image using an appropriate manual grey threshold (Fig. 1 (b_2)) by ImageJ software (Rasband 1997). The number of colloidal gold nanoparticles in the area of interest was obtained by particles counter ImageJ plugin applied in the EsB image.

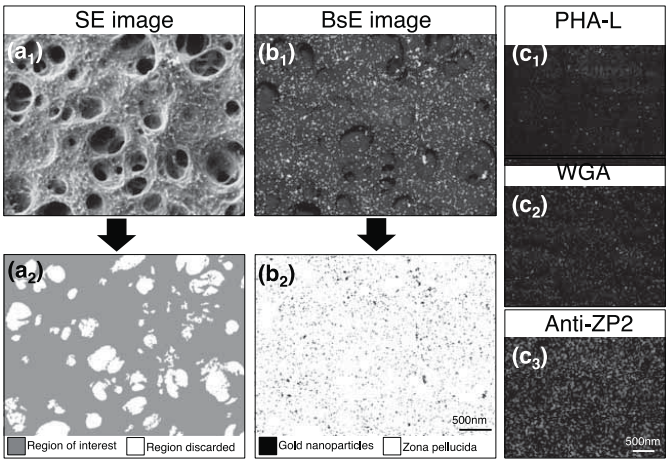

Fig. 1 Quantification of biomolecules binding sites at the ZP surface under field emission scanning electron microscope using gold conjugates. The secondary electron detector (SE) was used to capture an image of the ZP surface ($\mathbf{a_1}$), from which the region of interest was determined ($\mathbf{a_2}$). The backscattered electron in-lens detector (BsE) was then used to visualize the gold nanoparticles ($\mathbf{b_1}$), which were counted ($\mathbf{b_2}$). Quantification of ZP binding sites was performed for PHA-L ($\mathbf{c_1}$), WGA ($\mathbf{c_2}$) and anti-ZP2 antibody ($\mathbf{c_3}$)

2.4 Embryo toxicity analysis

The potential toxicity of the anti-ZP2 antibody (sc32752, Santa Cruz Biotechnology) and the WGA and PHA-L lectins (W11262 and L32456, respectively, Invitrogen) on embryo preimplantation development was evaluated during *in vitro* culture at two different concentrations (10 and 100 μg/ml). Thus, three groups of PN embryos were cultured in parallel in KSOM supplemented with 10 or 100 μg/ml of each biomolecule and their development was compared with that of a control group cultured in KSOM without any of the three biomolecules. The development stage of the four embryo groups was assessed every 24 h up to the blastocyst stage (96 h of culture).

2.5 Biofunctionalization of microspheres and attachment to the zona pellucida

Polystyrene microspheres of 6 μm in diameter and presenting either carboxyl (18141-2 Fluoresbrite YG Carboxylate Microspheres, Polysciences Inc., USA) or amino (19118-2 Polybead Amino Microspheres, Polysciences Inc.) reactive groups were used as model microparticles, due to their simple and efficient biofunctionalization using commercially available kits. Lectins (WGA or PHA-L) and the anti-ZP2 antibody were covalently coupled to carboxylate (COOH) and amino (NH_2) microspheres, respectively, using the highest amount of protein recommended (400 μg) for the protein coupling kits to saturate the available binding sites (24350-1 PolyLink Protein Coupling Kit for carboxylate Microspheres, 19540-1 Glutaraldehyde Kit for Amino Beads, Polysciences Inc.) and following the manufacturer's protocol. According to the technical data sheet of the protein coupling kit, it is estimated that an amount of 2.38 picograms of protein was bound per biofunctionalized microsphere. Thus, the estimated number of molecules bound per microsphere was 3.76×10^7 WGA molecules, 1.12×110^7 PHA-L molecules, or 9.54×110^6 anti-ZP2 antibody molecules.

To test the capacity of the three types of biofunctionalized microspheres to attach to the ZP of oocytes and embryos and to remain attached during oocyte activation (zona reaction) and *in vitro* embryo culture, oocytes and PN embryos were rolled over biofunctionalizated microspheres on a culture dish until 10 microspheres were attached to each of them. PN embryos were immediately transferred to KSOM medium and cultured for 96 h, whereas oocytes were parthenogenetically activated by a 6 min exposure to 7 % ethanol in KSOM-H and incubated in KSOM culture media supplemented with 5 μg/ml cytochalasin B for 6 h to prevent second polar body extrusion. Oocytes were then washed extensively and those showing two visible pronuclei were cultured in KSOM for 96 h. The developmental stage and the number of microspheres attached to each fertilized or parthenogenetic embryo were assessed every 24 h of culture. Embryos were cultured isolated into 15 μl microdrops to perform an individual monitoring.

2.6 Statistical analysis

Embryo viability rates were compared by Chi-squared or Fisher's exact tests. Results on microspheres retention and biomolecules ZP binding sites were compared using the Mann–Whitney and the Kruskal-Wallis tests for single and multiple comparisons, respectively. Probability values lower than 0.05 were considered as statistically significant.

3 Results

3.1 Binding of WGA, PHA-L and anti-ZP2 antibody to the zona pellucida

Alexa Fluor 594 conjugates were used to localize the distribution of WGA, PHA-L and anti-ZP2 antibody targets across the ZP of oocytes and PN embryos. Using a CLSM, equatorial XY fluorescence images were obtained and were overlapped with those acquired using the white field mode (Fig. 2). Whereas WGA was preferentially bound to the outer layer of the ZP (Fig. 2, (a_1) and (a_2)) and PHA-L was bound across the entire ZP, but showing a more intense signal on the inner layer (Fig. 2, (b_1) and (b_2)), the binding of anti-ZP2 antibody appeared uniform across the ZP thickness (Fig. 2, (c_1) and (c_2)). The binding pattern of each biomolecule was similar in both oocytes and PN embryos (Table 1).

3.2 Quantification of binding sites per area of zona pellucida

Quantification of ZP surface binding sites was performed in 15 oocytes and 15 PN embryos for each type of biomolecule (Table 1). Three zones, representing a total area of 37.5 μm^2, were randomly selected and analyzed for each specimen under FESEM. The anti-ZP2 antibody turned out to be the biomolecule with more binding sites per μm^2 of ZP (Fig. 1 (c_3)), both in oocytes and in PN embryos, though in this later group the value was significantly higher only when compared with that of PHA-L (Table 1). Regarding the two lectins, WGA (Fig. 1 (c_2)) had significantly more ZP binding sites than PHA-L (Fig. 1 (c_1)) both in oocytes and PN embryos. When comparing between oocytes and PN embryos, the number of ZP binding sites was similar both for anti-ZP2 and WGA, whereas for PHA-L it was significantly higher in oocytes than in PN embryos.

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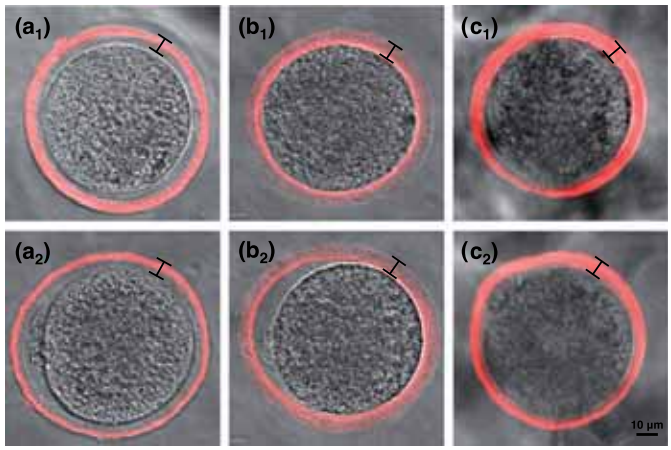

Fig. 2 Confocal laser scanning microscopy analysis of the ZP binding pattern for the three biomolecules tested. Equatorial XY fluorescence detection of WGA (**a**), PHA-L (**b**), and anti-ZP2 antibody (**c**) by the Alexa-594 conjugates of these molecules (red fluorescence) shows their distribution across the ZP of oocytes ((**a**$_1$) – (**c**$_1$)) and PN embryos ((**a**$_2$) – (**c**$_2$)). The bar indicates the distance between the inner and the outer surfaces of the ZP

3.3 Toxicity of free anti-ZP2 antibody, WGA and PHA-L in embryo development

The toxicity of the three types of biomolecules during embryo culture was tested at concentrations of 10 and 100 μg/ml. For each concentration, at least 50 PN embryos, divided in three replicates, were cultured in KSOM culture media supplemented with WGA, PHA-L or anti-ZP2 antibody. In parallel, a total of 100 control embryos were cultured in the absence of the biomolecules. Embryos cultured in the presence of the anti-ZP2 antibody invariably died during the first 24 h of culture at both concentrations tested. The majority of embryos exposed to WGA underwent vacuolation and, only at 10 μg/ml, a small proportion (18 %) was able to cleave, but failed to develop any further. In contrast, embryos cultured in the presence of PHA-L developed normally, following the same timing of cleavage and achieving an equivalent blastocyst rate (10 μg/ml: 88 %; 100 μg/ml: 88 %) as control embryos (86 %).

Table 1 Qualitative and quantitative analysis of the binding of different types of biomolecules to the ZP of mouse oocytes and embryos

Group	Biomolecule	Location of binding sites in the ZP	Number of binding sites/μm^2±SE
Oocytes	WGA	Outer	159.69±20.45^a
	PHA-L	Inner	5.57±0.35^{b*}
	Anti-ZP2	Across	334.60±29.37^c
PN Embryos	WGA	Outer	153.56±13.94^a
	PHA-L	Inner	2.15±0.29^{b*}
	Anti-ZP2	Across	303.95±37.11^a

$^{a, b, c}$ Different superscript denote significant differences between rows of the same group ($p<0.05$)

* Denote significant differences between groups for the same biomolecule ($p<0.05$); SE: standard error

3.4 Attachment of biofunctionalized microspheres to the zona pellucida of oocytes and embryos

Biofunctionalized microspheres were first imaged under CLSM to evaluate the biofunctionalization process (Fig. 3 (a)). As expected, all biofunctionalized microspheres showed red fluorescence at their periphery, corresponding to the Alexa Fluor 594 biomolecule conjugated to lectins or to the secondary antibody (Alexa Fluor 594 conjugated to the rabbit anti-rat IgG) used to detect the anti-ZP2 antibody.

Next, for each type of biomolecule tested, a group of 50 fertilized embryos with 10 biofunctionalized microspheres attached (Fig. 3, (b_1) and (b_2)), divided in three replicates, were followed during 96 h of culture. Their development stage and the mean number of microspheres attached to their ZP were evaluated every 24 h in order to determine the potential toxicity of the biomolecules bound to the microspheres surface and the stability of the microsphere attachment to the ZP surface, respectively. In addition, to determine the stability of the microsphere attachment during the zona reaction, a group of 60 oocytes with 10 biofunctionalized microspheres attached was parthenogenetically activated and then cultured for 96 h and evaluated every 24 h.

When oocytes and embryos were moved between drops of medium after the attachment of the microspheres to their ZP (e.g. for oocyte activation, post-activation washes or final transfer of activated oocytes and fertilized embryos to culture drops), some microspheres detached from the ZP. Because of this, the mean number of microspheres attached per embryo at 0 h of culture was lower than 10 in all experimental groups (Table 2). Consistent with the higher number of drop changes that parthenogenetically activated embryos underwent in comparison with fertilized embryos, mean values at this time point were lower in parthenogenetic than in fertilized embryos, though the difference was only

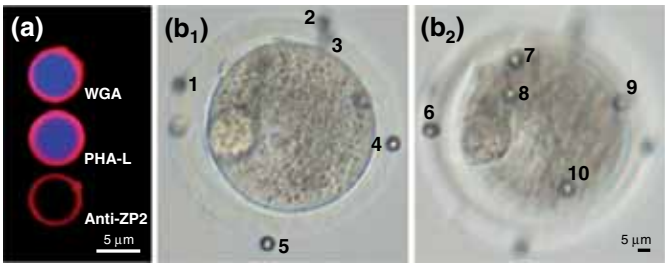

Fig. 3 Biofunctionalization of microspheres and attachment to the ZP. COOH microspheres (WGA and PHA-L microspheres) were autofluorescent (blue). The red fluorescence emission at the periphery of the microspheres corresponds to the Alexa-594 WGA, PHA-L or anti-ZP2 antibody conjugates (**a**). Biofunctionalized micospheres were able to attach to the oocytes/embryos ZP (**b**). Different focal planes ((**b**$_1$) and (**b**$_2$)) of the same PN embryo allow to visualize the 10 microspheres initially attached to its ZP

significant for anti-ZP2-microspheres. In fact, the binding of anti-ZP2-microspheres to the ZP seemed to be less robust than the binding of the other two types of biofunctionalized microspheres, as their retention at 0 h of culture was significantly lower when compared to those of WGA- or PHA-L ones, both in parthenogenetic and in fertilized embryos.

In parthenogenetic embryos, comparison between 0 and 24 h of culture, when the changes in ZP composition are supposed to take place due to the artificial activation of the oocytes, revealed no significant differences in the mean number of microspheres attached for any of the three types of biofunctionalized microspheres. However, anti-ZP2-microspheres showed again a significantly lower retention than lectin-biofunctionalized ones. A similar trend was observed in fertilized embryos: at 24 h, mean values of microspheres attached were equivalent to those found at 0 h of culture for all types of biofunctionalized microspheres, and anti-ZP2-microspheres showed the lowest retention, though only significant in the case of WGA. As observed at 0 h of culture, the mean number of anti-ZP2-microspheres attached to the ZP was significantly lower in parthenogenetic than in fertilized embryos.

By 96 h of culture, all experimental groups achieved a blastocyst rate equivalent to that of their corresponding control group (Table 2). Both in parthenogenetic and in fertilized embryos, the mean number of WGA-microspheres attached to their ZP was equivalent to that found at the start of the culture period (0 h), and significantly higher than the mean number of PHA-L- and anti-ZP2-microspheres that remained attached by 96 h of embryo culture. Therefore, WGA seems to provide a more stable and robust attachment of microspheres to the ZP both before (oocytes) and after (embryos) activation. The mean number of PHA-L-microspheres significantly decreased between 24 and 96 h of culture in both parthenogenetic and fertilized embryos, but that of anti-ZP2-microspheres was maintained. As observed at earlier time-points, the mean number of anti-ZP2-microspheres attached to the ZP at 96 h was significantly higher in fertilized than in parthenogenetic embryos.

It is important to note that microspheres processed with the biofunctionalization kits in the absence of WGA, PHA-L and anti-ZP2 antibody were not able to attach to the ZP of either oocytes or embryos.

Table 2 Microsphere retention and blastocyst rates of embryos with biofunctionalizated microspheres attached to their ZP

Group	Biomolecule	Mean number of microspheres/embryo±SE			Blastocyst rate (n)
		0 h	24 h	96 h	
Parthenogenetic embryos	None (control)	–	–	–	78.9 % (71/90)
	WGA	9.50±0.16^{a}	9.50±0.16^{a}	9.48±0.16^{a}	80.0 % (48/60)
	PHA-L	9.15±0.19^{a}	9.00±0.19^{a}	8.02±0.19^{b}	80.0 % (48/60)
	Anti-ZP2	7.38±0.21^{b*}	6.96±0.20^{b*}	6.64±0.18^{c*}	78.3 % (47/60)
Fertilized embryos	None (control)	–	–	–	88.8 % (71/80)
	WGA	9.81±0.08^{a}	9.70±0.09^{a}	9.56±0.10^{a}	86.0 % (43/50)
	PHA-L	9.53±0.11^{a}	9.20±0.16^{ab}	8.24±0.27^{b}	90.0 % (45/50)
	Anti-ZP2	8.56±0.25^{b*}	8.56±0.25^{b*}	8.14±0.25^{b*}	86.0 % (43/50)

$^{a, b, c}$ Different superscript denote significant differences between rows of the same column in the same group ($p<0.05$)

* Denote significant differences between groups for the same biomolecule in the same column ($p<0.05$); SE: standard error

4 Discussion

Traceability of oocytes and embryos requires their individual identification. Until now, efforts have been focused on providing an identifiable location for each specimen (Vajta et al. 2000; Gopichandran and Leese 2006; Somfai et al. 2010; Sugimura et al. 2010). However, the limitations of the existing methods led us to develop a novel system, in which the association of a specific encoded microparticle (barcode) to each oocyte/embryo would allow its unequivocal identification during the *in vitro* experimental procedures. In this sense, the microinjection of polysilicon barcodes into the perivitelline space was presented as a first approach (Novo et al. 2011), but the attachment of the barcodes to the embryo surface after hatching was an unexpected finding that needs to be avoided. In view of this, the association of the barcodes to the outer surface of the ZP was raised as feasible alternative, as it would prevent the direct contact between the barcodes and the embryo surface. To achieve this, the microparticle surface should be modified with a biomolecule capable of binding to the ZP. Among the biomolecules with this property, we have selected two lectins, WGA and PHA-L, which recognize different saccharides present in ZP, and one antibody, the anti-mZP2, which recognizes an epitope of mouse ZP2 glycoprotein. In order to determine which these three molecules is the most appropriate for the eventual biofunctionalization of the barcodes, once the oocyte/embryo tagging system is developed, in the present study we have used commercially available microspheres as a model because their functionalization is simple, fast and reliable using the protein coupling kits optimized by the manufacturer

The first parameter analyzed was the binding pattern of each biomolecule to the ZP both before (oocytes) and after fertilization (PN embryos). Using confocal microscopy, we showed that the three biomolecules displayed a different binding pattern across the ZP (outer side for WGA, inner side for PHA-L and across the whole thickness of the ZP for anti-mZP2 antibody), which was similar before and after fertilization (zona reaction). Our findings are in agreement with those of other authors who, using transmission electron microscopy (TEM), have described a uniform binding distribution of the anti-ZP2 antibody across the mouse ZP (Avilés et al. 1997; Jiménez-Movilla et al. 2004). Although the same authors also found a uniform binding pattern for the WGA lectin, a predominant binding of this lectin to the outer region of the ZP, as we have observed, has been reported in other studies (Nicolson et al. 1975). Therefore, according to our results, all three molecules are able to bind to the ZP, as expected, but PHA-L would be the less appropriate candidate for the eventual biofuntionalization of barcodes since the majority of its targets seem to be located in the inner side of the ZP.

Whereas confocal analysis allowed us to determine the location of the biomolecules binding sites across the ZP, quantification od these sites required the use of electron microscopy techniques, and FESEM in particular was used for this purpose. Despite ZP cytochemistry has been extensively studied by TEM (El-Mestrah and Kan 2002; Jiménez-Movilla et al. 2004 and 2009), no studies on ZP surface cytochemistry have been performed by scanning electron microscopy, to our knowledge. On the other hand, immunogold labeling has been recently applied to analyze the presence of a specific molecule present in the plasma membrane of a cell using FESEM (Muscariello et al. 2008), but our study is the first to apply this technique to analyze the ZP of oocytes/embryos. As used in the present study, FESEM allows to quantify the number of targets at the ZP surface for a particular biomolecule conjugated with gold nanoparticles, and at the same time to observe the ultrastructure of the sample. Because only binding sites present at the surface of the ZP are accessible for biomolecules once they have been covalently linked to a microparticle, it was relevant for our study to quantify the binding sites for WGA, PHA-L and anti-ZP2 antibody at the ZP surface by FESEM and to determine whether there are differences among biomolecules, and between oocytes and embryos. As it is known, the biochemical composition of the ZP changes after oocyte fertilization or artificial activation due to the secretions of cortical granules, which lead to the zona reaction (Wassarman 1987a, b). The released proteases during this process promote, among other modifications, the cleavage of ZP2 (120 kDa) to ZP2f (90 kDa) (Moller and Wassarman 1989). However, according to our results, this modification does not prevent the binding of the anti-ZP2 antibody to its epitope, as the number of ZP binding sites recognized was equivalent between oocytes and embryos. Glycosidases are also released by cortical granules during the zona reaction, but, according to our results, only the saccharides recognized by PHA-L seem to be significantly altered by these glycosidases, resulting in a more than two-fold decrease in the number of binding sites in PN embryos when compared to oocytes, whereas the number of binding sites for WGA are preserved after fertilization. This difference between both lectins may be attributed to the complexity of the saccharide recognized by PHA-L (Hammarström et al. 1982), which may render it more susceptible to modifications during the zona reaction than the saccharide recognized by WGA (Nagata and Burger 1974). Although in the present study no differences were found in the number of ZP binding sites between oocytes and embryos for WGA and the anti-mZP2 antibody, TEM cytochemistry studies performed by others in ultrathin sections of mouse embryos ZP showed a significant decrease in the number of binding sites for both biomolecules in the outer region of the ZP after fertilization (Avilés et al. 1997).

Despite the anti-mZP2 antibody (first) and WGA (second) were the biomolecules with the highest numbers

of binding sites per ZP area, both were toxic for the embryos when used as culture media supplements at any of the two concentration tested, whereas PHA-L was not. Our results with the two lectins agree with those described by Menino et al. (1989). Nevertheless, it is important to point out that when the biomolecules were covalenty linked to microspheres, their detrimental effect was avoided. Thus, microspheres biofunctionalized with these biomolecules were not toxic for the embryos, indicating that the covalent attachment of the biomolecule to the microspheres surface is stable, preventing the release of the biomolecule from the microparticle, its diffusion across the ZP and its contact with the embryo.

The biofunctionalization of the microspheres also allowed us to analyze the ability of each tested biomolecule to efficiently attach microparticles to the ZP of oocytes and embryos and to maintain them attached after the zona reaction (parthenogenetic activation) and during *in vitro* development. Despite the highest numbers of ZP binding sites were observed for the anti-ZP2 antibody, the microspheres biofunctionalized with this biomolecule detached easier from the ZP than lectin-biofunctionalized microspheres after the initial manipulations (observations at time of 0 h), indicating a weaker initial binding than that achieved by lectins. This observation was more evident in the parthenogenetic embryos, because they underwent many more manipulations than fertilized ones. As expected from our previous results on the quantification of binding sites in oocytes and PN embryos, ZP biochemical modifications occurring during oocyte activation did not affect microsphere attachment when WGA or the anti-ZP2 antibody were used for biofunctionalization (observations at 24 h in parthenogenetic embryos). And, surprisingly, even though the number of ZP binding sites for PHA-L was reduced in PN embryos when compared to oocytes, the mean number of PHA-L-biofunctionalized microspheres attached to the oocytes ZP was maintained after their activation.

Differences in the strength and stability of microsphere attachment to the ZP promoted by the different biomolecules tested can be attributed, in part, to the number of target binding sites that they present. Thus, whereas the anti-mZP2 antibody has two potential antigen-binding sites, lectins have four or more binding sites per biomolecule. Concretely, PHA-L has one carbohydrate binding site per monomer and, therefore, it has four sugar-binding sites (Sharon and Lis 1972). WGA is the only lectin with multiple carbohydrate binding sites per monomer (four), hence it has eight potential carbohydrate binding sites (Wright and Kellogg 1996), though only four have been detected functional at the same time (Wright and Kellogg 1996). These differences among biomolecules, together with our results on the quantification of binding sites in the outer surface of the ZP, explain the higher stability of WGA-biofunctionalized microspheres attachment to the ZP throughout the *in vitro* culture when compared to PHA-L- and, especially, anti-ZP2-biofunctionalized microspheres (observations at 96 h).

In conclusion, WGA lectin is, among the three candidates tested, the most efficient biomolecule to biofunctionalize microspheres with the aim to attach them to the ZP of mouse oocytes and embryos, and to maintain them attached through zona reaction and embryo *in vitro* culture up to the blastocyst stage. Moreover, as the saccharides recognized by WGA are highly abundant in the ZP of most mammalian species (Skutelsky et al. 1994; Habermann et al. 2011), the same WGA-biofunctionalized microparticles would be able to attach to the ZP of oocytes/embryos from other mammalian species, including humans and farm animals. The immediate future is the biofuncionalization of polysilicon barcodes with the WGA lectin in order to tag and track mammalian oocytes/embryos with the aim of reducing the risk of mismatching errors.

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Articulo III (Objetivos 2 y 3):

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

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human reproduction

ORIGINAL ARTICLE *Embryology*

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.

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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 (Schnauffer *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 Katlic, 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, Ramaderia 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.

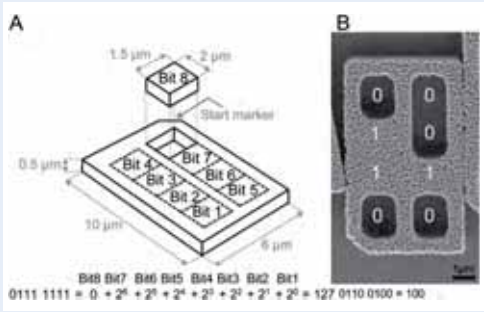

Figure 1 Design and dimensions of polysilicon barcodes. (**A**) Schematic representation of the barcodes used, showing shape, dimensions, number of bits and the start marker. (**B**) Scanning electron microscope image of a representative barcode, in which the binary code number is indicated. The corresponding conversion of the binary code to the decimal system is detailed below each image.

The fabrication process has been previously described (Fernández-Rosas *et al.*, 2009; Novo *et al.*, 2011).

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_2SO_4: H_2O_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_4OH:H_2O_2:H_2O (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-aminoethoxy)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.

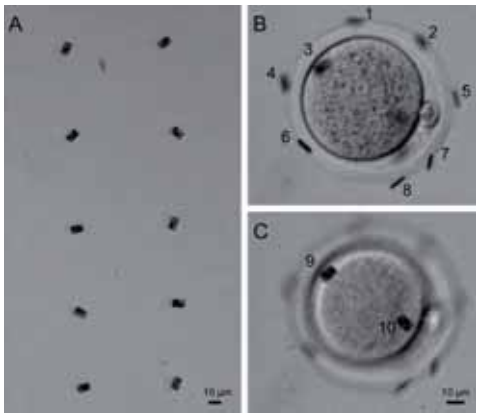

Figure 2 Embryo tagging by the attachment of 10 WGA-biofunctionalized barcodes to the ZP outer surface. (**A**) A group of 10 barcodes strategically distributed in two rows (five barcodes per row) at the bottom of a manipulation medium drop to ensure, after rolling an embryo over them, an equidistant distribution of the barcodes around the ZP surface. (**B** and **C**) Two different focal planes of the same embryo with a total of 10 barcodes attached to the outer surface of its ZP.

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-Farmavic, 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; Fig. 2A). A distance of 60 ± 5 µm was kept between each barcode to ensure, later, an equidistant distribution of the barcodes around the ZP surface. Next, the dish containing the barcodes was placed under a stereoscopic microscope and a single embryo was transferred into each drop. Each embryo was then rolled over the barcodes by means of a mouth-controlled aspiration

system, until the 10 barcodes were attached to its ZP (Fig. 2B and C). As the mere contact between the barcodes and the ZP surface was enough to allow their attachment, it took only ~20 s to tag each embryo. Finally, each tagged embryo was transferred to a microdrop of KSOM(aa) under mineral oil (M8410, Sigma, USA) in a 60 mm tissue culture dish (Z721034, Sigma, USA), and individually cultured at 37° in a 5% CO_2 atmosphere for 96–120 h in parallel to a group of control non-tagged embryos.

Cultured embryos were monitored every 24 h to assess their developmental progression. In the case of tagged embryos, the number of barcodes remaining attached to the ZP of the developed embryos (mean retention) and the number of developed embryos in which at least one barcode of the total attached could be clearly read under the inverted microscope, and therefore the embryo could be successfully identified, were also recorded (200× magnification; identification rate). It is important to note that, in order to simulate an automatic barcode reading process, the reading of the barcodes was performed only by adjusting the focus on the inverted microscope, without embryo manipulation. Therefore, only embryos with at least one barcode in the correct orientation were successfully identified (Fig. 2B and C). Finally, blastocysts were kept in culture until they hatched and the number of hatched blastocysts that were completely free of the barcodes was determined (release rate).

Embryo transfer

After 24 h in culture, a group of 2-cell stage tagged embryos and a group of control non-tagged embryos were separately transferred into 0.5 day *post coitum* recipient females (10–12 embryos/female). Naturally ovulating CD-1 female mice aged between 8 and 12 weeks and mated with vasectomized males of the same strain, to induce pseudopregnancy, were used as recipients. After embryo transfer, females were kept in individual cages and were allowed to deliver naturally.

Embryo freezing and thawing

Tagged embryos which had cleaved to the 2-cell stage and retained the 10 attached barcodes after 24 h in culture 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% CO_2 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 χ^2 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.

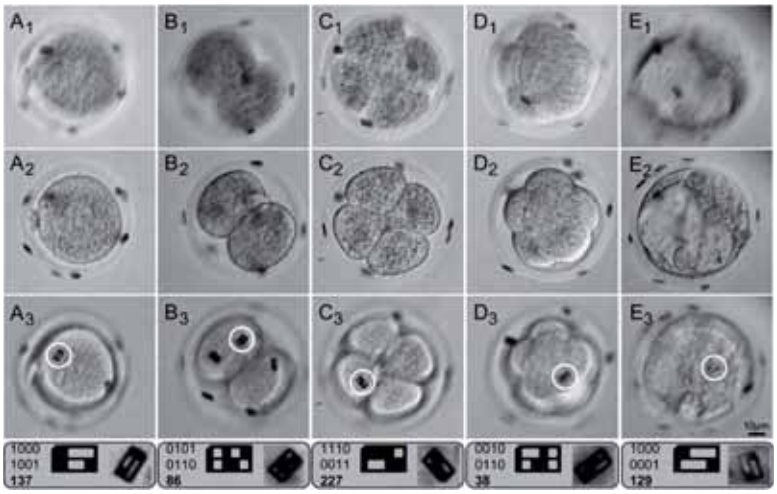

Figure 3 *In vitro* development of tagged embryos and identification process. Three different focal planes of 1-cell (**A_1–A_3**), 2-cell (**B_1–B_3**), 4-cell (**C_1–C_3**), compacting 8-cell (**D_1–D_3**) embryos and a hatching blastocyst (**E_1–E_3**) with barcodes attached to their ZP: identification 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, its diagrammatic representation and the corresponding conversion of the binary code to the decimal system (in bold) are detailed in the box below each image series.

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

Number of barcodes per embryo	Tagged embryos	Developed embryos with barcodes (%)				
		0 h	24 h	48 h	72 h	96 h
10	140	124 (88.6)	121 (88.3)	108 (80.0)	93 (71.5)	83 (65.9)
9		13 (9.3)	13 (9.5)	21 (15.6)	29 (22.3)	33 (26.2)
8		2 (1.4)	2 (1.5)	5 (3.7)	6 (4.7)	8 (6.3)
7		1 (0.7)	1 (0.7)	1 (0.7)	2 (1.5)	2 (1.6)
≤6		0	0	0	0	0
Mean retention ± SEM*	10[a]	9.86 ± 0.04[a,b]	9.85 ± 0.04[a,b]	9.75 ± 0.05[b,c]	9.64 ± 0.06[c,d]	9.56 ± 0.06[d]

SEM, standard error of the mean.
*Mean number of barcodes attached per embryo.
[a–d]Different superscripts denote significant differences among mean retention values at different time points ($P < 0.05$).

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

Number of barcodes per embryo	Developed embryos successfully identified (%)					
	0 h	24 h	48 h	72 h	96 h	Total
10	88/88 (100)	81/83 (97.6)	72/75 (96.0)	64/66 (96.9)	59/59 (100)	364/371 (98.1)[a]
9	8/9 (88.9)	12/12 (100)	17/17 (100)	21/22 (95.5)	24/25 (96.0)	82/85 (96.5)[a]
8	1/2 (50.0)	2/2 (100)	5/5 (100)	5/6 (83.4)	6/8 (81.8)	19/23 (82.6)[b]
7	0/1 (0)	1/1 (100)	1/1 (100)	1/2 (50.0)	1/2 (50.0)	4/7 (57.1)[b]
Total	97/100 (97.0)	96/98 (98.0)	95/98 (96.9)	91/96 (94.8)	90/94 (95.7)	469/486 (96.5)

No significant differences among total identification rates at different time points were detected.
[a,b]Values with different superscripts significantly differ ($P < 0.05$).

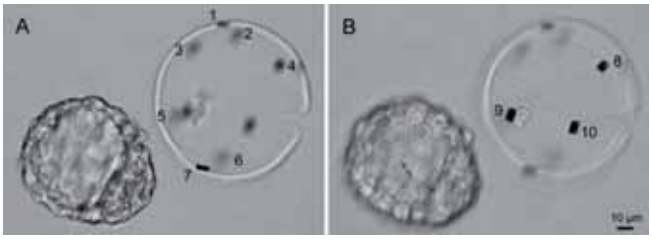

Figure 4 Barcode release after blastocyst hatching. Two different focal planes (**A** and **B**) of a hatched blastocyst free of barcodes and of the corresponding empty ZP with the 10 barcodes attached to its outer surface.

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

Number of barcodes per embryo	2-cell embryos cryopreserved	Developed embryos with barcodes (%)			
		0 h	24 h	48 h	72 h
10	84	19 (23.5)	16 (21.6)	15 (21.1)	15 (21.1)
9		27 (33.3)	26 (35.1)	20 (28.2)	18 (25.4)
8		18 (22.2)	19 (25.7)	22 (31.0)	22 (31.0)
7		12 (14.8)	10 (13.5)	11 (15.5)	12 (16.9)
6		4 (5.0)	2 (2.7)	2 (2.8)	3 (4.2)
5		1 (1.2)	1 (1.4)	1 (1.4)	1 (1.4)
≤4		0	0	0	0
Mean retention ± SEM*	10[a]	8.52 ± 0.14[b]	8.56 ± 0.13[b]	8.45 ± 0.14[b]	8.38 ± 0.14[b]

SEM, standard error of the mean.
*Mean number of barcodes attached per embryo.
[a,b]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 IV Identification rates of frozen–thawed tagged embryos at different time points during *in vitro* culture.

Number of barcodes per embryo	2-cell embryos cryopreserved	Developed embryos successfully identified (%)				
		0 h	24 h	48 h	72 h	Total
10	80/84 (95.2)	18/19 (94.7)	15/16 (87.5)	15/15 (100)	15/15 (100)	143/149 (96.0)[a]
9		25/27 (92.6)	26/26 (100)	20/20 (100)	18/18 (100)	89/91 (97.8)[a]
8		15/18 (83.3)	17/19 (89.5)	19/22 (86.4)	21/22 (95.5)	72/81 (88.9)[a]
7		11/12 (91.7)	9/10 (90.0)	10/11 (90.9)	10/12 (83.3)	40/45 (88.9)[a,b]
6		2/4 (50.0)	2/2 (100)	1/2 (50.0)	2/3 (66.7)	7/11 (63.7)[b,c]
5		0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)	1/4 (25.0)[c]
Total	80/84 (95.2)	71/81 (87.7)	69/74 (93.3)	65/71 (91.5)	67/71 (94.4)	352/381 (92.4)

No significant differences among total identification rates at different time points were detected.
[a–c]Values with different superscripts significantly differ ($P < 0.05$).

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. The 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 (Novo *et al.*, 2011). 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 ~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 one 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 ± 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

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.

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Artículo IV (Objetivos 3 y 4):

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

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BARCODE TAGGING OF HUMAN OOCYTES AND EMBRYOS TO PREVENT MIX-UPS IN ASSISTED REPRODUCTION TECHNOLOGIES

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Running title: Barcode tagging of human oocytes and embryos

ABSTRACT

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 embryos during assisted reproduction technologies?

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 is totally effective and several mix-up cases have been reported worldwide. Our group has previously developed an effective direct embryo tagging system in the mouse, 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 or intracytoplasmic sperm injection (n=205) were donated by patients (n=76) undergoing assisted reproduction technologies. *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 intracytoplasmic sperm

injection and parthenogenetic activation. Experiments were conducted from January 2012 to January 2013.

Participants/materials, setting, methods: Barcodes were fabricated in polysilicon and biofunctionalizated 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 propidum 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 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 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 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.

Limitations, reasons for caution: Although the direct embryo tagging system developed is effective, it only allows the identification and traceability of oocytes destined to intracytoplasmic sperm injection and embryos. Thus, the traceability of all reproductive samples (oocytes destined to *in vitro* fertilization, semen) is not yet ensured.

Wider implications of the findings: The direct embryo tagging system developed here provides fertility clinics with a novel tool to reduce the risk of mix-ups in human assisted reproduction technologies.

Study funding/competing interest(s): Study supported by the *Sociedad Española de Fertilidad*, the Spanish Ministry of Education and Science (TEC2011-29140-C03) and the *Generalitat de Catalunya* (2009SGR-00282 and 2009SGR-00158). The authors do not have any competing interest.

Keywords: polysilicon barcodes, traceability, embryo identification

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 concerns 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 United Kingdom (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 (*Matcher*^{TM},IMT, UK) or radio frequency identification labels (*IVF Witness*^{TM}, 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 microinjection 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 cells 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

111 terms of development rates to the blastocyst stage, morphokinetic parameters assessed by time-
112 lapse monitoring, and embryo quality through morphological grading as well as blastocyst cell
113 counting after differential staining. For the second purpose, retention of the barcodes attached to
114 the ZP and embryo identification rates were examined at several time-points during *in vitro* culture,
115 after vitrification-warming procedures, and after a simulated oocyte intracytoplasmic sperm
116 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 failed to be fertilized after conventional *in vitro* fertilization (IVF) were used. These oocytes (n=21), retrieved from 5 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 37ºC and 6% CO_2 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 to 255).

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 ZP 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 ZP. 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 ZP. The simple contact between the WGA-biofunctionalized

barcodes and the ZP outer surface was enough to allow their attachment, and it required only approximately 20 s per each 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 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 37ºC and 6% CO_2. Oocytes were cultured in G1-HSA at 37ºC and 6% CO_2 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 at 37ºC and 6% CO_2 from day 1 to day 3. At day 3, embryos were washed with G-MOPS and transferred to G2 medium (Vitrolife, Sweden), both mediums supplemented with HSA. At 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 into 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 timings 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 to calculate the time-points and durations of the developmental events analyzed. As embryos used in our study came from both ICSI and conventional IVF

194 procedures and the exact timing of their fertilization was unknown, thawing was considered as the
195 start point and the time of all events is expressed as hours post-thawing.
196
197 **Embryo morphological grading**
198 Embryos were graded on days 2, 3, 5 and 6 of their *in vitro* development according to the criteria for
199 the morphological evaluation of human oocytes, early embryos and blastocysts proposed by the
200 *Asociación para el Estudio de la Biología de la Reproducción* (2008). The evaluation was performed
201 on acquired images and embryos were categorized in four grades, from A (embryo with optimal
202 quality and the best implantation potential) to D (a poor-quality embryo with a low chance of
203 implantation).
204 Day 2 and day 3 embryos were assigned to a particular category depending on the number of cells,
205 symmetry and granularity of the blastomeres, percentage of fragmentation, presence of
206 multinucleated blastomeres and presence of vacuoles. Blastocysts were categorized based on the
207 day of formation (day 5: categories A and B; day 6: categories C and D) and the inner cell mass (ICM),
208 trophectoderm (TE) and blastocoele morphologies.
209
210 **Blastocyst evaluation by differential staining**
211 The cellular composition of day 6 blastocysts was assessed by differential staining of ICM and TE cells
212 using a modification of the protocol described by Thouas et al (2001). Briefly, blastocysts were first
213 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 33258 Hoechst (Sigma, Spain) and stored in this solution at 4ºC 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 BioPharma Co., Japan). After warming, blastocysts were transferred to droplets of G2-HSA under mineral oil and cultured at 37ºC and 6% CO_2 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,

234 cell counts after blastocyst differential staining and morphokinetic parameters were compared

235 between tagged and control embryos using either Mann-Whitney or Student's t-test, as appropriate.

236 A p-value < 0.05 was considered statistically significant.

237 **RESULTS**

238 **Tagging system evaluation during *in vitro* embryo culture**

239 To test the validity and safeness of the tagging system in human embryos, 58 pronuclear stage
240 embryos were tagged, in five experimental replicates, by the attachment of 10 WGA-
241 biofunctionalized barcodes to the outer surface of their ZP. Then, they were cultured *in vitro* for 6
242 days (120 h) in separate microdrops, to monitor each embryo individually (Fig. 2), in parallel to a
243 group of control non-tagged embryos (n=51). The development rates of tagged and control embryos
244 were equivalent at all stages, and a similar blastocyst rate was achieved in the two groups (46.7%,
245 27/58 tagged embryos; 47.1%, 24/51 control embryos).
246 The number of barcodes remaining attached to the ZP of the developed tagged embryos (mean
247 retention) was recorded every 24 h (Table I). Only 3 embryos (5.2%) lost one of the barcodes while
248 being transferred from the drops where tagging was performed to the drops of culture medium (0 h,
249 day 1 of culture). Significant losses were neither detected after media change at day 3 (48 to 72 h)
250 and day 5 (96 to 120 h). However, a small number of barcodes were progressively lost during
251 embryo culture and after 72 h of culture (day 4) the mean retention value, despite being high (9.54 ±
252 0.12 barcodes per embryo), became significantly lower than the initial number of barcodes attached
253 per embryo (10). No other significant decrease in the mean retention value was observed during the
254 rest of the culture. In spite of these losses, after 120 h of *in vitro* culture 37% of the embryos still
255 maintained the total of 10 barcodes initially attached and the mean retention value was as high as
256 9.22 ± 0.13 barcodes per embryo. More important, 100% of the developed embryos maintained at

257 least 8 barcodes attached to their ZP at the end of the culture (120 h). Yet, after embryo hatching
258 the barcodes remained attached to the empty ZP and 100% of the hatched embryos were free from
259 barcodes.
260 The number of developed tagged embryos in which at least one barcode could be read under the
261 inverted microscope (200x magnification; identification rate) was also recorded every 24 h. It is
262 important to note that, to mimic an eventual automatic barcode reading process, the dish
263 containing the embryos was placed under an inverted microscope and the reading of the barcodes
264 was performed only by adjusting the focus, without any embryo manipulation. Hence, only embryos
265 with at least one barcode in a correct spatial orientation for reading could be successfully identified
266 (Fig. 2).
267 Total identification rates were high and similar at all time points analyzed (90.9% - 100%; Table II). As
268 expected, they decreased as the number of barcodes attached per embryo decreased, though
269 significant differences were only observed in embryos with 8 or less barcodes. Regardless of the
270 number of barcodes attached per embryo and the culture time-point, embryo identification was
271 successful in 96.9% of the total analyses realized (n=256) under the conditions used in our study.
272 However, it is important to point out that 100% of the embryos could be successfully identified
273 simply by rotating them to allow the correct spatial orientation of at least one of their barcodes.
274 Embryo quality was also assessed in this first set of experiments, using two different quality assays:
275 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 any of the two quality assays applied.

Morphokinetic evaluation of tagged embryos

In a second set of experiments (6 replicates), one-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 V). 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 (120h, day 6; Table I and Table 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).

296 **Tagging system evaluation after vitrification/warming procedures**

297 To test the validity of the tagging system after a vitrification process, tagged (n=21) and control (n=
298 19) day 5 and 6 expanded blastocysts produced in the second set of experiments were vitrified once
299 their morphokinetic evaluation was completed. The number of barcodes attached to each tagged
300 blastocyst was recorded before vitrification, and a mean number of 9.48 ± 0.16 barcodes/blastocyst
301 was observed (Table VI). After warming, blastocysts were analyzed at 3 h and 24 h to determine the
302 number of re-expanded blastocysts, which was equivalent between tagged and control blastocysts.
303 Moreover, both for control and for tagged embryos, re-expansion rates were equivalent between
304 day 5 and day 6 vitrified blastocysts at both time-points analyzed (Table VI).
305 Barcode retention mean values and embryo identification rates were also calculated at both time-
306 points after warming. Vitrification and warming procedures led to a significant decrease in the mean
307 number of barcodes attached per embryo (Table VI). However, after warming, no more significant
308 barcode losses occurred during culture, and at least 7 (range: 7 - 10) and 5 (range: 5 - 9) barcodes
309 per embryo remained attached after 24 h of culture in day 5 and day 6 warmed blastocysts,
310 respectively. At the end of the culture, the mean barcode retention value in vitrified-warmed
311 blastocysts (7.79 ± 0.35; Table VI) was significantly lower than that obtained in the first set of
312 experiments in non-vitrified blastocysts (9.22 ± 0.13; Table I).
313 As observed in non-vitrified embryos, the identification rate of vitrified-warmed embryos was
314 directly related to the number of barcodes that remained attached to their ZP, decreasing as the
315 number of barcodes decreased (83.3% - 91.7%; Table III). However, because the integrity of all the

316 barcodes was preserved during the vitrification-warming procedures and all the embryos had at
317 least 3 barcodes attached after warming, an identification rate of 100% could be achieved simply by
318 rotating the non-identifiable embryos until one of the remaining barcodes was properly oriented for
319 reading.
320
321 **Tagging system evaluation after oocyte intracytoplasmic injection and parthenogenetic activation**
322 Immature and mature oocytes (n=21) tagged with 10 barcodes were microinjected without sperm to
323 simulate an ICSI procedure (Figure 3) and the number of barcodes that remained attached after
324 microinjection was recorded to evaluate the possible effects of this manipulation on barcode
325 retention. Only one of the oocytes lost one barcode during the micromanipulation process, so the
326 mean barcode retention was 9.95 ± 0.05 barcodes per oocyte, with an identification rate of 100%.
327 Then, mature oocytes were selected (n=13) and parthenogenetically activated. The number of
328 barcodes remaining attached to the ZP of the activated oocytes (20 h post-ionomycin exposure) was
329 again recorded, as an indicator of the possible effects on barcode retention of the ZP modifications
330 that take place during the zona reaction. Nine of the mature oocytes were successfully activated, as
331 judged by the presence of pronuclei 20 h later, and all of them retained all the barcodes during the
332 activation process.

333 **DISCUSSION**

334 In the present work, the applicability in human oocytes and embryos of a direct tagging system
335 previously developed by our group in mouse embryos (Novo et al., 2013a) was tested. First, we
336 evaluated the safeness of the tagging system by assessing potential effects on the *in vitro*
337 development and quality of human embryos. The development rates of tagged embryos were
338 equivalent at all stages to those of control embryos. Similarly, the quality of the tagged embryos, as
339 judged by their morphological grading at several developmental stages and by the cell counts in day
340 6 blastocysts, was equivalent to that of control embryos. For a more exhaustive and precise analysis
341 of embryo competence, a morphokinetic evaluation of tagged embryos by time-lapse monitoring
342 was also performed. This evaluation revealed no alterations in the timing and duration of several
343 developmental events in the tagged embryos that achieved the blastocyst stage, when compared to
344 control embryos, corroborating the results of our other tests of embryo developmental potential
345 and quality. Therefore, according to all the analyses performed, tagging of human embryos with
346 WGA-biofunctionalized barcodes does not have any significant effect on embryo developmental
347 potential and quality, in agreement with our previous results in mouse and bovine embryos (Novo et
348 al. 2013 a,b).
349 In parallel to the safeness of the system, we also investigated its effectiveness for the labeling and
350 identification of human oocytes and embryos. For this purpose, some of the key laboratory
351 procedures typically conducted during the course of an ART cycle were carried out with tagged
352 oocytes and embryos, and the retention of the barcodes on the ZP as well as the identification rates

353 of the tagged samples at several points of the procedures were determined. A small, though
354 significant, progressive loss of barcodes occurred during embryo culture, as we had previously
355 observed in mouse embryos (Novo et al., 2013a). This loss was not caused by embryo manipulations
356 during sequential culture, and could probably be attributed to a slight weakening of the WGA-ZP
357 binding resulting from the modifications that normally occur in the ZP during early embryo
358 development (Vanroose et al., 2000). Despite these losses, barcode mean retention values at the
359 end of the culture were high (9.22 ± 0.13 barcodes/embryo), and equivalent to those previously
360 observed in mouse tagged embryos (9.56 ± 0.06 barcodes/embryo) (Novo et al., 2013a). High
361 retention values of WGA-biofunctionalized barcodes were indeed expected, as a high affinity of
362 WGA to the ZP of both mouse (Skutelsky et al., 1994; Novo et al., 2013c) and human (maymon et al.,
363 1994; Talevi et al., 1997; Jiménez-Movilla et al., 2004) oocytes and embryos has been documented.
364 It is worth noting, however, that once the embryos hatched from the ZP all of them were freed from
365 barcodes, which remained attached to the empty ZP.
366 Retention of at least 7 barcodes per embryo during culture facilitated the achievement of a very high
367 global identification rate (96.9%), which was also similar to the rate obtained in our earlier study in
368 mouse embryos (96.5%) (Novo et al., 2013a). However, as previously pointed out, the simple
369 rotation of the non-identified embryos until one of their barcodes became properly oriented for
370 reading could have led to a 100% identification rate. We chose not to manipulate the embryos
371 during the identification process because, although manual eye reading of the barcodes was
372 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 which 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 ZP 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 ZP 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 ZP when embryos are not manipulated during the barcode reading process, a decrease in the barcode mean

393 retention values after vitrification-warming was paralleled by a decrease in embryo identification
394 rates when compared to non-cryopreserved blastocysts, though this decrease was not significant.
395 Therefore, the effectiveness of the tagging system is slightly reduced after embryo cryopreservation
396 but, again, it could be increased to 100% simply by rotating the embryos during the barcode reading
397 process or by the attachment of more than 10 barcodes per embryo.
398 Finally, the tagging system was also applied to human oocytes and its effectiveness during a
399 simulated ICSI procedure was tested. The presence of the barcodes on the oocyte ZP did not impair
400 the micromanipulation procedure in any way, and barcodes were seldom lost as a result of oocyte
401 manipulation and suctioning with the holding pipette. Therefore, tagging of oocytes would be totally
402 compatible with their subsequent fertilization by ICSI.
403 On the other hand, it is long known that the biochemical composition of the ZP changes after
404 fertilization due to the action of the glycosidases and proteases released from the cortical granules
405 in a process known as zona reaction (Wassarman, 1987). As this modification could potentially affect
406 the binding of WGA to the ZP and, therefore, the binding of the barcodes, we decided to artificially
407 induce the zona reaction in the tagged oocytes by parthenogenetic activation. Barcode detachment
408 was not observed in any of the activated oocytes, a result in agreement with previous studies by our
409 group in mouse oocytes demonstrating that the number of binding sites for the WGA lectin is
410 maintained after fertilization (Novo et al., 2013c). Thus, these observations support the applicability
411 and effectiveness of the direct tagging system for oocytes which have to be subsequently fertilized
412 by ICSI. Unfortunately, tagging would not be possible in oocytes destined to IVF, as they are not

413 denuded of cumulus cells and the ZP would not be free for barcode attachment. Another limitation
414 of the current system is the impossibility to tag sperm, another frequent source of ART mix-ups.
415 However, our group is presently working on the adaptation of the direct tagging method to human
416 sperm samples to overcome this limitation.
417 In conclusion, the applicability of the direct tagging system based on WGA-biofunctionalized
418 barcodes and previously developed in mice by our group has now been successfully proved in
419 human oocytes and embryos. The tagging system is simple, safe, and highly efficient, allowing the
420 identification of human oocytes and embryos during the various steps of an ART cycle. In a clinical
421 setting, each patient would be assigned a specific barcode number, and all her oocytes/embryos
422 would be tagged with this same barcode number. The tags would accompany the embryos
423 throughout the whole ART procedure and until hatching, so that embryos will be free of the
424 barcodes for implantation. The introduction of this direct tagging system in fertility clinics would be
425 straightforward, as no special or expensive equipment is needed, and would surely minimize the
426 occurrence of mix-ups. In fact, its application is compatible with the use of labware labeling systems
427 (either manual or electronic), so that both the labware and the biological samples could be tagged,
428 adding an extra level of security to the traceability control.

AUTHORS' ROLES

C.N, J.A.P, L.P.G and E.I obtained funding. 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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449 **CONFLICT OF INTEREST**

450 The authors declare no conflict of interest

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516

517 **FIGURE LEGENDS**

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

526

527 **Figure 2. *In vitro* development of tagged embryos and identification process.** Two different focal
528 planes of the same embryo observed on day 1 ($\mathbf{a_1}$ and $\mathbf{a_2}$), day 2 ($\mathbf{b_1}$ and $\mathbf{b_2}$), day 3 ($\mathbf{c_1}$ and $\mathbf{c_2}$), day 4
529 ($\mathbf{d_1}$ and $\mathbf{d_2}$), day 5 ($\mathbf{e_1}$ and $\mathbf{e_2}$) and day 6 ($\mathbf{f_1}$ and $\mathbf{f_2}$), with barcodes attached to its zona pellucida, are
530 shown. Identification was performed simply by adjusting the focus of the inverted microscope (200x
531 magnification) until a barcode properly oriented for reading was found (white circles). A magnified
532 image of the readable barcode (binary code= 0000 0000; decimal number= 0) is shown in the insets.
533 Scale bar = 20 μm.

534

535 **Figure 3. Intracytoplasmic sham injection of tagged oocytes.** (**a**-**d**) Image sequence of a tagged

536 oocyte during microinjection. (**e** and **f**) Two different focal planes of the oocyte after injection, with

537 the 10 barcodes still attached to its zona pellucida. Scale bar = 20 μm.

538

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

Number of barcodes per embryo	Number of tagged embryos	Number of developed embryos with barcodes (%)					
		0 h (day 1)	24 h (day 2)	48 h (day 3)	72 h (day 4)	96 h (day 5)	120 h (day 6)
10	58	55 (94.8)	45 (84.9)	35 (74.5)	25 (64.1)	13 (39.4)	10 (37.0)
9	-	3 (5.2)	7 (13.2)	10 (21.3)	11 (28.2)	14 (42.4)	13 (48.2)
8	-	0	0	1 (2.1)	2 (5.1)	5 (15.2)	4 (14.8)
7	-	0	1 (1.9)	1 (2.1)	1(2.6)	1 (3.0)	0
≤6	-	0	0	0	0	0	0
Mean retention ± SEM*	**10[a]**	**9.95 ± 0.03[a]**	**9.81 ± 0.07[a,b]**	**9.68 ± 0.09[a,b]**	**9.54 ± 0.12[b,c]**	**9.18 ± 0.14[c]**	**9.22 ± 0.13[c]**

*Mean number of barcodes attached per embryo; SEM: standard error of the mean.

[a-c] Different superscripts denote significant differences among mean retention values at different time-points ($p < 0.05$; Kruskal-Wallis, Dunn's *post hoc* test).

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

Number of barcodes per embryo	Number of developed embryos successfully identified (%)						
	0h (day 1)	**24h (day 2)**	**48h (day 3)**	**72h (day 4)**	**96h (day 5)**	**120h (day 6)**	**TOTAL**
10	55/55 (100)	44/45 (97.8)	35/35 (100)	25/25 (100)	13/13 (100)	10/10 (100)	182/183(99.5)[a]
9	2/3 (66.7)	6/7 (100)	10/10 (100)	11/11 (100)	13/14 (92.9)	13/13 (100)	55/57 (96.5)[a,b]
8	-	-	1/1 (100)	2/2 (100)	3/5 (60.0)	2/4 (50.0)	8/12 (66.7)[b]
7	-	1/1 (100)	0/1 (0)	1/1 (100)	1/1 (100)	-	3/4 (75.0)[b]
TOTAL	**57/58 (98.3)**	**51/53 (96.2)**	**46/47 (97.9)**	**39/39 (100)**	**30/33 (90.9)**	**25/27 (92.6)**	**248/256 (96.9)**

[a, b] Values with different superscripts significantly differ within the same column ($p < 0.05$; ANOVA).

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

Table III. Embryo grading after morphological evaluation of tagged and control embryos cultured in microdrops (Experiment 1) or in microwells (Experiment 2)

Experiment	Embryo group	Quality grade	Number of embryos (%)			
			Day 2	Day 3	Day 5	Day 6
1	Control	A	16 (31.4)	12 (29.4)	10 (66.7)	-
		B	19 (37.3)	3 (5.9)	5 (33.3)	-
		C	12 (23.5)	10 (19.6)	-	6 (66.7)
		D	4 (7.8)	23 (45.1)	-	3 (33.3)
	Tagged	A	15 (28.3)	10 (21.3)	11 (68.8)	-
		B	23 (43.4)	8 (17.0)	5 (31.2)	-
		C	11 (20.8)	6 (12.8)	-	6 (54.5)
		D	4 (7.5)	23 (48.9)	-	5 (45.5)
2	Control	A	6 (13.3)	6 (13.3)	5 (45.5)	-
		B	11 (24.4)	9 (20.0)	6 (54.5)	-
		C	13 (28.9)	4 (8.9)	-	6 (75.0)
		D	15 (33.3)	26 (57.8)	-	2 (25.0)
	Tagged	A	12 (25.0)	12 (25.0)	8 (66.7)	-
		B	12 (25.0)	4 (8.3)	4 (33.3)	-
		C	12 (25.0)	11 (22.9)	-	7 (77.8)
		D	12 (25.0)	21 (43.8)	-	2 (22.2)

No significant differences in quality grades were detected between control and tagged embryos ($p > 0.05$; Chi square for days 2 and 3, Fisher's exact test for days 5 and 6).

Table IV. Cell counts in day 6 blastocysts after differential staining.

Group	No. of blastocysts	Mean number of cells/blastocyst ± SEM*			ICM/TCN ratio ± SEM*
		TCN[a]	ICM[b]	TE[c]	
Control	24	100.9 ± 11.2	22.3 ± 1.9	78.5 ± 9.4	0.24 ± 0.01
Tagged	25	111.9 ± 13.6	24.7 ± 2.5	87.2 ± 11.3	0.23 ± 0.01

*SEM: standard error of the mean; aTCN: total cell number; bICM: inner cell mass; cTE: trophectoderm.

No significant differences were detected for any of the parameters analyzed between control and tagged blastocysts ($p > 0.05$; Mann-Whitney and Student's t-test).

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

Parameter analyzed	Time (h ± SEM (n))	
	Control	Tagged
Disappearance of pronuclei time-point	9.1 ± 0.6 (16)	8.2 ± 0.7 (19)
1st cytokinesis duration	0.45 ± 0.03 (19)	0.47 ± 0.05 (21)
2-cell stage time-point	13.9 ± 1.8 (19)	11.9 ± 0.8 (21)
Reappearance of nuclei after first cleavage time-point	15.2 ± 0. 9 (10)	16.1 ± 1.5 (13)
Reappearance of nuclei after first cleavage duration	3.0 ± 0.4 (10)	3.3 ± 0.5 (13)
3-cell stage time-point*	26.5 ± 2.8 (17)	24.6 ± 1.3 (18)
2-cell stage duration*	12.6 ± 0.6 (17)	12.7 ± 0.7 (18)
4-cell stage time-point	27.4 ± 2.4 (19)	26.2 ± 1.2 (21)
3-cell stage duration*	1.0 ± 0.2 (17)	1.3 ± 0.3 (18)
5-cell stage time-point	39.2 ± 2.6 (19)	41.8 ± 2.2 (21)
Compaction time-point	77.2 ± 1.9 (19)	75.2 ± 1.4 (21)
Morula time-point	85.4 ± 2.0 (19)	84.0 ± 1.7 (21)
Blastocyst time-point	94.8 ± 2.0 (19)	92.0 ± 1.9 (21)
Full Blastocyst time-point	104.1 ± 2.4 (19)	101.2 ± 2.3 (21)

SEM: standard error of the mean.

*Data for two control and three tagged embryos which divided directly from 1 to 3 cells are missing.

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 blastocysts.

Group		Vitrified			3 h post-warming			24 h post-warming		
		Blastocysts	Mean Retention*	ID (%)	Re-expanded (%)	Mean Retention*	ID (%)	Re-expanded (%)	Mean Retention*	ID (%)
Control	**Day 5**	11	-	-	8 (72.7)	-	-	10 (90.9)	-	-
	Day 6	8	-	-	6 (75.0)	-	-	6 (75.0)	-	-
	Total	19	-	-	14 (73.7)	-	-	16 (84.2)	-	-
Tagged	**Day 5**	12	9.67 ± 0.19^{a}	12 (100)	11 (91.7)	8.09 ± 0.41^{b}	10 (90.9)	12 (100)	7.92 ± 0.43^{b}	11 (91.7)
	Day 6	9	9.22 ± 0.28^{a}	8 (88.9)	6 (66.7)	7.83 ± 0.65^{b}	5 (83.3)	7 (77.8)	7.57 ± 0.61^{b}	6 (85.7)
	Total	21	9.48 ± 0.16^{a}	20 (95.2)	17 (81.0)	8.00 ± 0.34^{b}	15 (88.2)	19 (90.5)	7.79 ± 0.35^{b}	17 (89.5)

*Mean number of barcodes attached per embryo ± standard error of the mean (SEM).

[a, b] Values with different superscripts denote significant differences within the same group at different time-points ($p < 0.05$; Kruskal-Wallis, Dunn's *post hoc* test).

No significant differences in the identification rates of tagged blastocysts were detected among the different time-points ($p > 0.05$; ANOVA).

No significant differences in the re-expansion rates were detected between the control and the tagged groups ($p > 0.05$ Fisher's exact test).

Figure 1

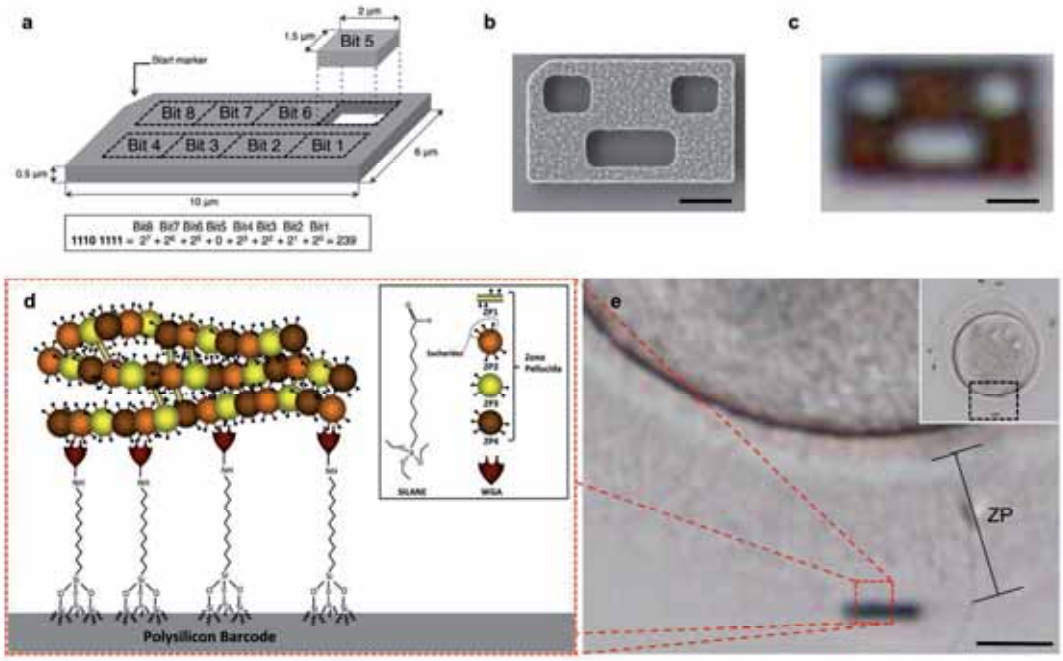

a
b
c
Bit 5
Bit 8
Bit 7
Bit 6
Bit 4
Bit 3
Bit 2
Bit 1
d
e
ZP
Polysilicon Barcode

Figure 2

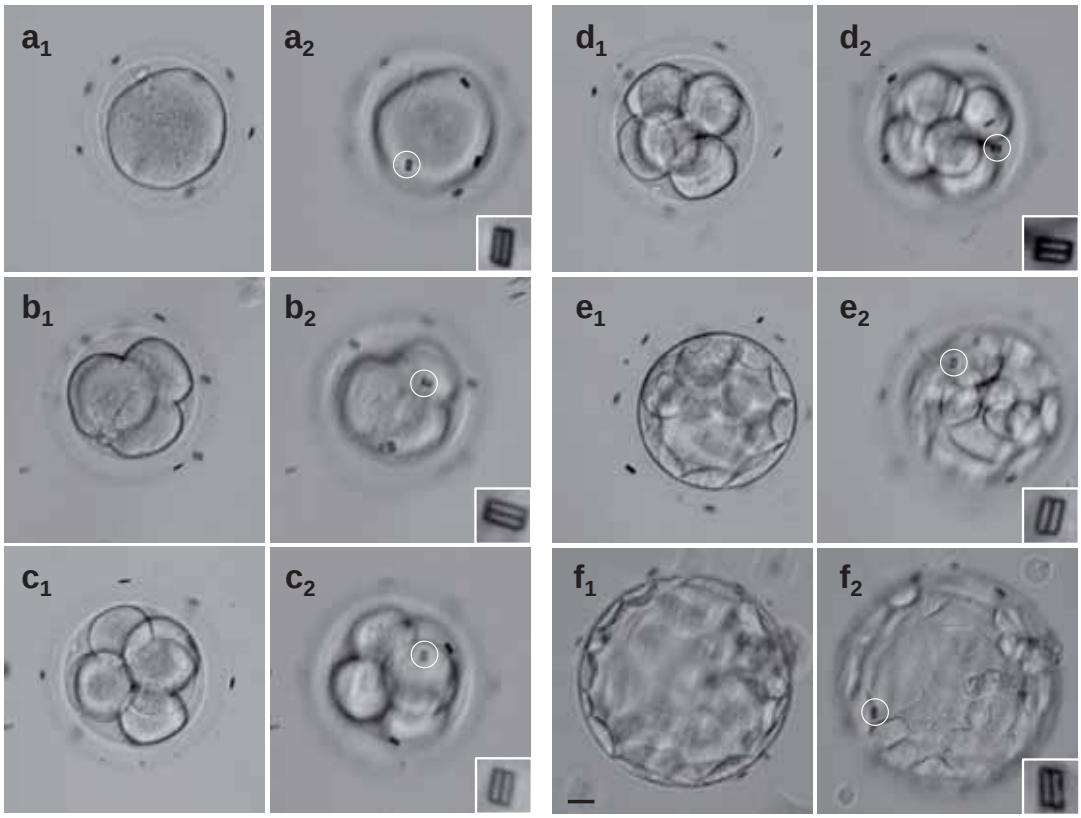
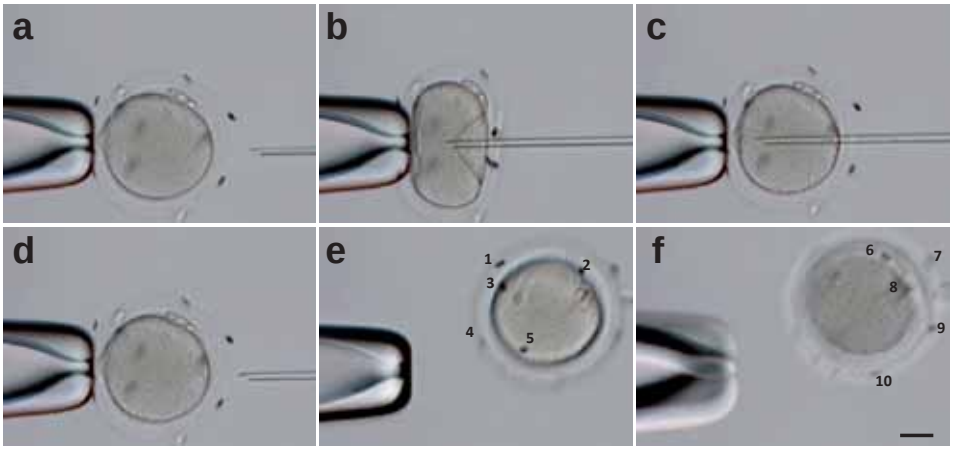


Figure 3



Artículo V (Objetivos 3 y 5):

"Identification of bovine embryos cultured in group by attachment of barcodes to the zona pellucida"

Autores: Sergi Novo, Roser Morató, Oriol Penon, Sara Durán, Leonardo Barrios, Carme Nogués, José Antonio Plaza, Luisa Pérez-García, Teresa Mogas, Elena Ibáñez.

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Identification of bovine embryos cultured in groups by attachment of barcodes to the zona pellucida

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Abstract. The low number of oocytes collected from unstimulated donors by ovum pick-up means that embryos produced from each individual female have to be cultured individually or in very small groups. However, it has been demonstrated that single-embryo culture is less efficient than embryo culture in groups. To overcome this limitation, we developed a direct embryo-tagging system, which allows the collective culture of embryos from different origins whilst preserving their pedigree. Presumptive bovine zygotes were tagged with eight wheat-germ agglutinin biofunctionalised polysilicon barcodes attached to the outer surface of the zona pellucida (ZP). Four different barcodes were used to encode groups of 20–25 embryos, which were then cultured in the same drop. Cleavage, Day-7 and Day-8 blastocysts and barcode retention rates were assessed. In addition, Day-7 blastocysts were vitrified and warmed. Barcode attachment to the ZP of bovine embryos affected neither *in vitro* embryo development nor post-warming survival of the tagged embryos. All the embryos maintained barcodes attached until Day 8 of culture (3.63 ± 0.37 barcodes per embryo) and could be identified. In conclusion, identification of embryos by barcodes attached to the ZP is feasible and will allow the culture of embryos from different donors in the same drop.

Additional keywords: collective culture, embryo tagging, microdevice, ovum pick-up, polysilicon.

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Introduction

Ultrasound-guided transvaginal follicular aspiration, or ovum pick-up (OPU), in combination with *in vitro* fertilisation (IVF), enables the production of embryos from live donor cattle of high genetic value. In order to preserve pedigree information, embryos produced from each oocyte donor must be cultured separately. In practice, as the number of cumulus–oocyte complexes (COCs) collected from a single cow during an OPU session is low (Petyim *et al.* 2003; Machado *et al.* 2006), this means that the resulting embryos must be cultured singly or in very small groups. Although some groups have achieved similar blastocyst rates by using collective and single-embryo culture systems (Carolan *et al.* 1996; Han *et al.* 2006), this later system is generally associated with low blastocyst rates and poor embryo quality (O'Doherty *et al.* 1997; Ward *et al.* 2000; Goovaerts *et al.* 2009). On the other hand, successful development of embryos cultured in groups has been extensively recognised in several species, and the benefits of collective culture are mainly attributed to autocrine–paracrine factors produced and secreted by embryos (Paria and Dey 1990; Moessner and Dodson 1995; Thibodeaux *et al.* 1995; O'Doherty *et al.* 1997; Fujita *et al.* 2006; Reed 2012; Richter 2008).

Co-culture with somatic cells improves blastocysts rates and quality of embryos cultured individually or in small groups (Donnay *et al.* 1997), as the somatic cells secrete embryotrophic factors and may also neutralise embryo toxic compounds (Orsi and Reischl 2007). However, as somatic cells may act as vectors for disease transmission (Le Tallec *et al.* 2001), their use in

commercial OPU–IVF settings is often banned for sanitary reasons. More recently, other culture systems aimed at improving embryo development in single or small-group culture conditions have been developed. Among these, 'apart together' culture systems (Goovaerts *et al.* 2010) are receiving increasing attention as they allow group stimulation and individual identification at the same time by physically separating a group of embryos sharing the same medium. They include the well-of-the-well (WOW) system (Vajta *et al.* 2000; Sugimura *et al.* 2010), in which embryos are cultured individually in microwells shaped on the bottom of the culture dish; the Cell-Tak system (Stokes *et al.* 2005; Gopichandran and Leese 2006), based on the use of a cell adhesive to attach the embryos to the bottom of the culture dish in a specific order; or the use of a polyester mesh composed of monofilaments between which embryos can be placed (Booth *et al.* 2007; Somfai *et al.* 2010). Although these systems allow the accumulation of beneficial autocrine factors and the dilution of metabolised toxic products (Vajta *et al.* 2000), all of them prevent the free movement of the embryos, interfering with the transmission of paracrine factors and facilitating the creation of local gradients resulting from embryo secretions or depletion of media components (Swain and Smith 2011; Smith *et al.* 2012). In addition, culture dishes must be handled very carefully, as a sudden movement of the dish may easily displace the embryos from their original location.

Here we report a novel *in vitro* culture system that allows the collective culture of embryos from different origins without movement restriction whilst preserving their pedigree identification during the whole culture. This system is based on the tagging of denuded zygotes with lectin-biofunctionalised polysilicon barcodes attached to the outer surface of the zona pellucida (ZP). In previous studies by our group, the biofunctionalisation of the polysilicon barcodes with wheat-germ agglutinin (WGA) lectin, which shows its highest affinity to N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid monosaccharides (Bhavanandan and Katlic 1979), abundant in the ZP of most mammalian species (Skutelsky *et al.* 1994; Habermann *et al.* 2011), has been proven as an efficient approach to achieve the attachment of the barcodes to the outer surface of the mouse ZP (Penon *et al.* 2012). Mouse pronuclear embryos tagged with the WGA-biofunctionalised barcodes could be successfully identified during *in vitro* culture and exhibited similar rates of *in vitro* and *in vivo* development as control non-tagged embryos (Novo *et al.* 2013).

In order to validate the developed methodology as a traceability system for bovine embryos cultured collectively, four different codifications of WGA-barcodes were used to tag presumptive zygotes obtained after IVF of slaughterhouse-derived oocytes. Zygotes tagged with the four barcode codifications were cultured together in groups of 20–25 into the same drop. The parameters evaluated were: (1) the effects of WGA barcodes attached to ZP on the *in vitro* development and the quality of the blastocysts produced; (2) the retention of WGA barcodes attached to ZP during culture up to blastocyst stage; (3) embryo identification by reading the barcodes under an inverted microscope and (4) the effects of WGA barcodes on blastocyst survival after vitrification-warming procedures.

Materials and methods

Unless otherwise indicated, all reagents were purchased from Sigma (Madrid, Spain).

Barcode design, fabrication and biofunctionalisation

Barcodes are three-dimensional polysilicon microparticles of 10 µm in length and 6 µm in width and with a thickness of 0.5 µm. They have an asymmetric corner to offer a start-reading marker, and carry eight rectangular bits with 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 (Novo *et al.* 2011, 2013). In the present study, only four types of barcodes, with the decimal codifications 0, 105, 153 and 255 were employed (Fig. 1).

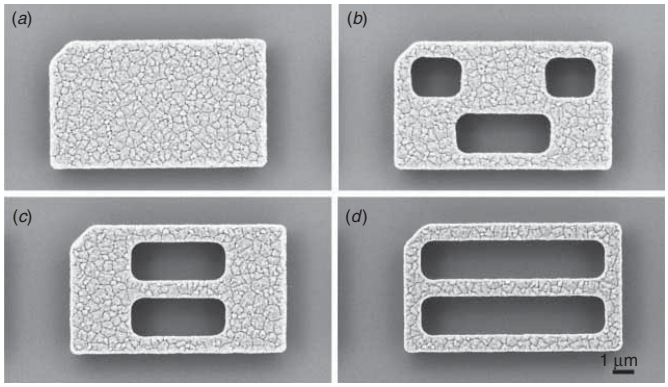

Fig. 1. Polysilicon barcodes. Scanning electron microscope images of the barcodes with decimal codification of (*a*) 255, (*b*) 105, (*c*) 153 and (*d*) 0.

Barcodes were fabricated on 4″ p-type (100) silicon wafers using silicon microtechnologies used for microelectromechanical systems fabrication as previously described (Fernández-Rosas *et al.* 2009; Novo *et al.* 2011).

The biofunctionalisation of polysilicon barcodes with the WGA lectin (Invitrogen, Grand Island, NY, USA) followed the same protocol as has been previously described (Penon *et al.* 2012).

In vitro *embryo production*

The techniques used to produce bovine embryos *in vitro* have been previously described in detail (Rizos *et al.* 2002). Briefly, COCs were obtained by aspirating 2- to 10-mm follicles from bovine ovaries collected at a slaughterhouse. Groups of 50 COCs were matured for 24 h in TCM-199 medium supplemented with 10% (v/v) fetal calf serum (FCS), 10 ng mL^{-1} epidermal growth factor and 50 μg mL^{-1} gentamicin at 38.5°C under an atmosphere of 5% CO_2 in air with maximum humidity. For IVF, the COCs were transferred, in groups of up to 50, to four-well plates containing 250 μL of fertilisation medium per well (25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg mL^{-1} fatty acid-free BSA and 10 mg mL^{-1} heparin–sodium salt; Calbiochem, Darmstadt, Germany). Motile spermatozoa were obtained by centrifuging frozen–thawed spermatozoa through a discontinuous BoviPure density gradient (Nidacon International, Norrköping, Sweden), counted in a haemocytometer and diluted in an appropriate volume of fertilisation medium to give a final concentration of 2×10^6 spermatozoa mL^{-1}. A 250-μL aliquot of this suspension was then added to each fertilisation well (final concentration 1×10^6 spermatozoa mL^{-1}). Plates were incubated at 38.5°C under an atmosphere of 5% CO_2 in air with maximum humidity for ~20 h. Finally, presumptive zygotes were vortexed for 3 min to remove cumulus cells.

Embryo tagging, culture and monitoring

After denudation, presumptive zygotes were tagged by the attachment of eight WGA-biofunctionalised barcodes to the outer surface of their ZP. First, an aliquot of each of the four types of barcodes was deposited into a separate drop of G-MOPS (Vitrolife, Göteborg, Sweden). Next, a group of five to seven presumptive zygotes were transferred to each of the four drops and each presumptive zygote was individually rolled over the barcodes, under a stereomicroscope and by means of a mouth-controlled aspiration system, until eight barcodes were attached to its ZP. As the simple contact between the barcode and the ZP surface was enough to attach barcodes, it took only ~20 s to tag each presumptive zygote.

Presumptive zygotes tagged with the four types of barcodes were mixed and cultured together in groups of 20–25 in 25-μL drops of synthetic oviductal fluid (aaSOF; Holm *et al.* 1999) supplemented with 5% FCS (v/v) in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. A group of non-tagged presumptive zygotes was cultured in parallel in the same conditions and served as controls. Cleavage rate at 48 h post-insemination (hpi) and blastocyst yield at Days 7 and 8 of *in vitro* culture were recorded. In the case of tagged embryos, the number of barcodes remaining attached to their ZP was assessed at each of these time points. In addition, the number of Day-7 blastocysts in which at least one barcode could be clearly read under the inverted microscope (200× magnification) was also recorded (identification rate).

Blastocyst vitrification and warming

Day-7 blastocysts from the control and tagged groups were vitrified and warmed using the cryotop device (Kitazato BioPharma, Fuji, Japan) and vitrification and warming solutions described by Kuwayama *et al.* (2005). The holding medium (HM) for formulating all vitrification and warming solutions consisted of Hepes-buffered TCM199 with 20% (v/v) FCS. Blastocysts were first incubated for 9–15 min in the equilibration solution (ES) consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulfoxide (DMSO) in HM. They were then transferred to the vitrification solution (VS) containing 15% (v/v) DMSO, 15% (v/v) EG and 0.5 M sucrose dissolved in HM. After incubating for 30–40 s, the blastocysts were loaded onto the cryotop, the solution was removed leaving only a thin layer covering the blastocysts, and the sample was quickly plunged into liquid nitrogen. The entire process from exposure to VS to plunging was completed within 90 s. Loaded cryotops were stored in liquid nitrogen.

All warming steps were performed at 38.5°C. The cryotop was directly immersed in the warming solution containing 1 M sucrose dissolved in HM. After 1 min, the recovered blastocysts were transferred to the dilution solution, which contained 0.5 M sucrose dissolved in HM. The blastocysts were incubated for 3 min and cryoprotectant diffusion out of the embryo was promoted by gentle pipetting. Next, the blastocysts were incubated in washing solution (WS = HM) for 5 min. After a final rinse again in WS for 1 min, blastocysts were transferred to aaSOF culture medium and incubated at 38.5°C in a 5% O_2 and 90% N_2 atmosphere. Embryo survival was assessed under a stereomicroscope according to the extent of re-expansion of the blastocysts and their ability to hatch after 3 and 24 h.

Blastocyst evaluation by nuclear staining

The cellular composition of control and tagged Day-8 blastocysts, both after fresh culture and after vitrification–warming procedures, was assessed by differential staining of inner cell mass (ICM) and trophectoderm (TE) cells as described by Thouas *et al.* (2001). Briefly, blastocysts were first incubated in PBS with 1% (v/v) Triton X-100 and 100 μg mL^{-1} propidium iodide for up to 30 s. Blastocysts were then immediately transferred into a fixative solution of 100% ethanol supplemented with 25 μg mL^{-1} Hoechst 33258 and stored in this solution at 4°C overnight. Fixed and stained whole blastocysts were mounted onto a slide in a 3-μL drop of Vectashield (Vectorlabs, Burlingame, CA, USA) and flattened with a coverslip. Cell counting was performed using the projection images obtained from xyz hyperstack scan by an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Hamburg, Germany). The overlay image resulting from the capture of the two channels showed ICM and TE nuclei as blue and red-pink, respectively.

Statistical analysis

All experiments were repeated seven times. Data were analysed using the GraphPad (GraphPad Software Inc., La Jolla, CA, USA) and SPSS (IBM, New York, NY, USA) software.

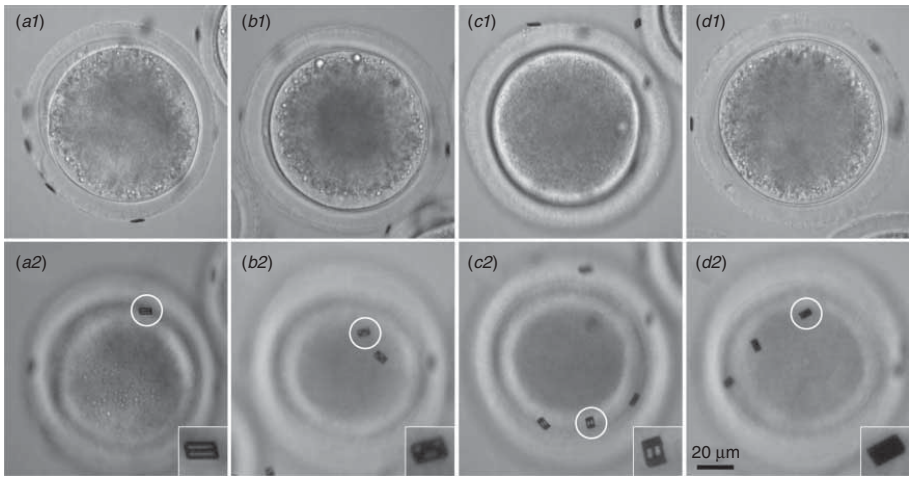

Fig. 2. Presumptive bovine zygotes tagged with WGA-biofunctionalised barcodes attached to their ZP. Two different focal planes of presumptive zygotes tagged with barcodes (*a1*, *a2*) 0, (*b1*, *b2*) 105, (*c1*, *c2*) 153 or (*d1*, *d2*) 255 are shown. Identification was performed simply by adjusting 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 is shown in the insets.

Table 1. *In vitro* development of tagged bovine embryos

Results are expressed as mean ± s.e.m. No significant differences between the control and the tagged groups were detected for any of the parameters analysed

Group	No. of embryos examined	No. of embryos cleaved (%)	No. of embryos developed to the blastocyst stage (%)	Number of cells per Day-8 blastocyst			Ratio of ICM to total cells (%)
				Total (*n*)	Inner cell mass (ICM)	Trophectoderm	
Control	309	231 (74.3 ± 4.2)	92 (31.0 ± 4.3)	92.4 ± 6.5 (25)	25.6 ± 2.1	66.8 ± 4.7	27.8 ± 0.9
Tagged	315	248 (77.2 ± 4.5)	114 (34.8 ± 4.6)	87.7 ± 6.7 (27)	23.1 ± 1.6	64.6 ± 5.3	26.8 ± 0.8

Development rates and vitrified–warmed blastocyst survival rates were analysed using a mixed linear model. Embryo cell numbers were analysed by Student *t*-test. The values of barcode retention were compared using Kruskal–Wallis and Dunn tests. Significance was set at $P < 0.05$.

Results

In vitro *development of tagged embryos*

A total of 315 presumptive zygotes were tagged with eight barcodes each (Fig. 2) and cultured in groups of 20–25, in parallel to 309 non-tagged (control) presumptive zygotes. Development rates showed no significant differences between tagged and control groups at any of the time points analysed (Table 1). Significant differences were not detected between the two groups in the total, ICM and TE cell numbers in Day-8 blastocysts (Table 1).

To determine the effectiveness of the tagging system, the mean number of barcodes that remained attached per developed embryo was evaluated. A significant proportion of the eight barcodes initially attached were detached mainly during the first 24 h of culture, so at 48 hpi the mean number of barcodes per embryo was reduced to 5.01 ± 0.12 (modal number = 4; Table 2). The mean number of barcodes in Day-7 and Day-8 blastocysts was 4.11 ± 0.19 (modal number = 4) and 3.63 ± 0.37 (modal number = 3), respectively (Table 2). It is important to remark that at all time points of analysis 100% of the developed embryos maintained at least one barcode attached to their ZP surface (Table 2). Thus, 100% of the embryos could be successfully identified under the inverted microscope, either by simply adjusting the focus or, when none of the barcodes were feasible to read, by rotating the embryo until the correct orientation of at least one of the barcodes was achieved (Fig. 3).

Vitrification and warming survival of tagged blastocysts

Up to three blastocysts were loaded per cryotop with a total of 82 tagged and 66 non-tagged (controls) Day-7 blastocysts vitrified. The re-expansion rates achieved by these two groups at 3 and 24 h after warming were not significantly different (Table 3). After 24 h of culture, re-expanded embryos were differentially stained and no significant differences were observed in the total number of cells per blastocyst or in the ratio of ICM to total cells between tagged and non-tagged groups (Table 3).

Table 2. Barcode retention during *in vitro* embryo culture
[a,b,c]Values with different superscripts differ significantly ($P < 0.05$)

Day after insemination	No. of embryos	Number of developed embryos with x number of barcodes attached (%)								Mean retention
		1	2	3	4	5	6	7	8	
Day 1	315								315 (100)	8.00 ± 0.00^{a}
Day 2	248	7 (2.8)	19 (7.7)	27 (10.9)	51 (20.6)	40 (16.1)	42 (16.9)	35 (14.1)	27 (10.9)	5.01 ± 0.12^{b}
Day 7	82	3 (3.7)	12 (14.6)	16 (19.5)	20 (24.4)	15 (18.2)	8 (9.8)	5 (6.1)	3 (3.7)	4.11 ± 0.19^{c}
Day 8	32	6 (18.8)	4 (12.5)	7 (21.9)	6 (18.8)	3 (9.4)	2 (6.2)	2 (6.2)	2 (6.2)	3.63 ± 0.37^{c}

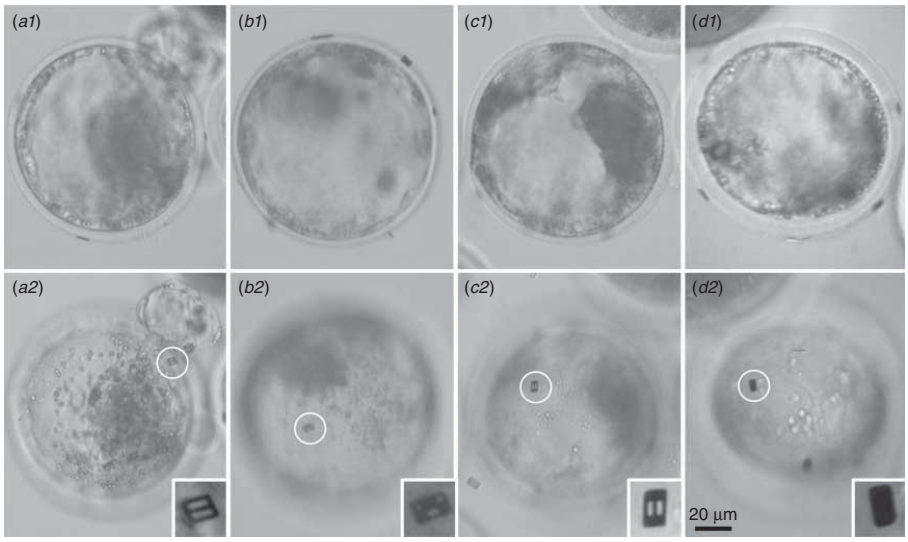

Fig. 3. Bovine blastocysts tagged with WGA-biofunctionalised barcodes attached to their ZP. Two different focal planes of blastocysts tagged with barcodes (*a1*, *a2*) 0, (*b1*, *b2*) 105, (*c1*, *c2*) 153 or (*d1*, *d2*) 255 are shown. Identification was performed simply by adjusting the focus of the inverted microscope (200× magnification) until a barcode properly oriented for reading was found (white circles). A magnified image of the readable barcode is shown in the insets.

Table 3. Post-warming re-expansion of tagged bovine embryos
Results are expressed as mean ± s.e.m. No significant differences between the control and the tagged groups were detected for any of the parameters analysed

Group	No. of Day-7 blastocysts	No. of re-expanded blastocysts at		Number of cells per blastocyst			Ratio of ICM to total cells (%)
		3 h (%)	24 h (%)	Total (*n*)	Inner cell mass (ICM)	Trophectoderm	
Control	66	55 (89.2 ± 5.3)	57 (89.6 ± 4.0)	132.8 ± 5.4 (51)	30.8 ± 0.9	102.1 ± 4.7	23.4 ± 0.5
Tagged	82	65 (81.2 ± 6.3)	66 (81.5 ± 4.9)	143.7 ± 6.4 (57)	31.6 ± 1.1	112.1 ± 5.5	22.7 ± 0.5

Discussion

Traceability of cultured bovine embryos is key in certain applications, such as in commercial OPU–IVF procedures, although the developmental potential of embryos cultured singly or in very small groups is compromised (O'Doherty *et al.* 1997; Ward *et al.* 2000; Goovaerts *et al.* 2009). In the present work, we describe a novel *in vitro* culture system that allows the collective culture of bovine embryos using the traditional drop-culture approach, but preserving their identification during the whole culture period. This system is based on the tagging of denuded zygotes with WGA-biofuntionalised barcodes attached to the ZP. Our group has previously developed and tested this tagging system in mouse embryos (Novo *et al.* 2013), and now the application of this technology in the culture of bovine

embryos is presented. The tagging process is simple and fast. It can be performed under a stereoscopic microscope, with a mouth- or hand-controlled aspiration system, and requires only ~20 s to attach eight barcodes to each embryo. After tagging each group of presumptive zygotes with a specific barcode codification, they can be cultured together in the same drop of medium, as their pedigree information is maintained through their distinctive barcodes. Embryos can be easily and quickly (at most 20 s per specimen) identified by the simple reading of the barcode under an inverted microscope.

To test the validity of the system in bovine embryos, *in vitro*-produced zygotes from slaughterhouse-derived oocytes were used as a model. Collective *in vitro* culture of tagged embryos was first carried out. No detrimental effects of the attached WGA-biofunctionalised barcodes on the developmental potential of the tagged embryos up to the blastocyst stage were observed, as expected according to our previous studies with non-biofunctionalised polysilicon microparticles (Fernández-Rosas *et al.* 2009, 2010; Novo *et al.* 2011). In addition, although free WGA lectin has shown embryo toxicity (Menino *et al.* 1989), we have demonstrated that covalent attachment of WGA to the barcodes surface by an efficient biofuntionalisation protocol prevents its toxic effects (Penon *et al.* 2012). Further, we have also proven that WGA-biofunctionalised barcodes attached to the ZP of mouse embryos do not affect either *in vitro* nor *in vivo* development of the tagged embryos (Novo *et al.* 2013). Thus, in agreement with these previous results, the development rates and the quality of the tagged bovine embryos in the present study were equivalent to those of the control non-tagged embryos.

Even though all WGA-biofunctionalised barcodes were able to attach to the ZP of the presumptive zygotes during the tagging process, some of them detached during the *in vitro* culture, as previously observed in tagged mouse embryos (Novo *et al.* 2013). Because of this, eight barcodes were attached to each embryo, with the aim that at least one would remain attached up to the blastocyst stage and allow the identification of the embryos up to the end of the culture period. Indeed, barcode detachment was more evident in bovine embryos (present study) than in mouse embryos (Novo *et al.* 2013), suggesting that the mouse ZP may have more superficial binding points available to WGA than the bovine ZP. This observation is in agreement with the reported ZP-binding pattern of this lectin in the two species: in mice, WGA shows a predominant binding to the outer region of the ZP (Nicolson *et al.* 1975), whereas in cattle it shows a preferential binding to the inner part (Habermann *et al.* 2011). Other molecules more efficient for barcode attachment to the bovine ZP could be investigated in further studies. Is important to note that barcodes that detached from a tagged embryo during culture were not able to reattach to the ZP of another embryo, and they just lay at the bottom of the culture drop.

Despite some barcode losses, all bovine embryos maintained at least one barcode attached to their ZP up to the blastocyst stage, which was enough to permit their correct identification. Although the system described here allows the identification of the embryos at any time of culture, we only performed embryo identifications at the end of the *in vitro* culture as, in the bovine species, the blastocyst is the optimum stage for transfer or cyropreservation (Lamb 2005; Morató *et al.* 2010). So in a real OPU–IVF procedure, the blastocyst would be the stage at which embryos would be removed from culture and should need to be identified. Embryo identification was performed, quickly and easily, by manual eye reading of the barcodes under inverted microscope. As 100% of the embryos maintained at least one barcode attached to their ZP until the end of culture, 100% of the blastocysts could be identified, simply by rotating the embryos until one of the barcodes became properly oriented for reading. We are currently developing a computer program for the automatic reading of the barcodes, which will allow a faster and more reliable reading just by placing the tagged embryos under the inverted microscope and capturing an image of one of its barcodes.

With recent advances in cryopreservation protocols, cryopreservation of blastocysts has become a readily-available and reliable tool with which to preserve embryos that have shown the best *in vitro* developmental potential (Ménézo 2004). In addition, the ability of embryos to survive cryopreservation has been used by many as an indicator of embryo quality and viability (Rizos *et al.* 2008). Likewise, total, TE and ICM cell numbers and the ICM ratio in blastocysts are commonly-used criteria for assessment of blastocyst quality (VanSoom *et al.* 1996). Thus, the ability of tagged and identified blastocysts to undergo cryopreservation procedures, especially vitrification, and blastocyst cell numbers were evaluated. After warming, the blastocyst re-expansion rate at 3 and 24 h, the number of cells and the ICM ratio were equivalent between tagged and non-tagged embryos, confirming the similar quality of the two groups of embryos.

The novel traceability system presented here has several advantages over the current systems developed with this same aim. First, our system does not require a physical modification of the culture support, as the WOW and Cell-Tak systems do, which is labour intensive and time consuming (Matoba *et al.* 2010). Second, embryos tagged with the WGA-biofunctionalised barcodes remain identifiable throughout the whole culture period, even after medium changes or embryo manipulations. These are critical points in the preservation of the traceability in the current systems. Third, because identification of the embryos does not rely on their position in the culture drop, as in the 'apart together' systems, the embryos remain identifiable even after they are accidentally displaced by an abrupt movement of the dish (Goovaerts *et al.* 2010; Somfai *et al.* 2010).

In conclusion, the bovine embryo-tagging and identification system designed in this study is effective and harmless for the embryos and it allows the culture of embryos from different origins in the same drop, keeping the benefits of collective culture in the efficient traditional culture system. Moreover, in contrast to other culture systems based on the embryo position in the culture drop for identification purposes, such as the current 'apart together' systems, the WGA-barcode tagging system could be combined with the emergent culture platforms of dynamic culture, which have reported promising results on *in vitro* embryo production (Smith *et al.* 2012). Although the collective culture of embryos from different donors is not always permitted for commercial use and export, this new embryo-tagging and identification system may improve the

efficiency of OPU–IVF procedures and facilitate the production of embryos from live animals of high genetic merit.

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4. DISCUSIÓN

Los sistemas de etiquetaje actualmente utilizados para la identificación de muestras reproductivas en las TRAs para humanos y animales de renta presentan ciertas limitaciones. Con la finalidad de proporcionar sistemas de etiquetaje que permitan una identificación más segura, en esta tesis se ha desarrollado un sistema de etiquetaje directo de la muestra reproductiva (ovocitos y embriones) basado en el uso de códigos de medidas micrométricas (microcódigos) fabricados en polisilicio. La puesta a punto de este sistema ha contemplado la selección de un modelo de microcódigo óptimo entre varios diseños, el desarrollo de un método de vinculación directa entre microcódigo y muestra eficiente, y la valoración de la inocuidad y efectividad del sistema de etiquetaje y la aplicabilidad de dicho sistema en muestras de origen humano y bovino.

4.1. Selección del diseño de microcódigo para el etiquetaje directo de ovocitos y embriones.

A partir de tres diseños diferentes de microcódigo (tipo A, tipo B y tipo C) se propuso seleccionar el más apropiado para el etiquetaje e identificación de embriones de ratón (modelo utilizado). El etiquetaje de los embriones se realizó utilizando técnicas de micromanipulación, mediante las cuales los microcódigos fueron depositados en el espacio perivitelino (EPV). La presencia de los microcódigos en el EPV de los embriones no alteró su potencial de desarrollo *in vitro* hasta el estadio de blastocisto. Estos resultados eran de esperar ya que con anterioridad nuestro grupo había demostrado la inocuidad de micropartículas de silicio y polisilicio internalizadas tanto en macrófagos humanos como en embriones de ratón (Fernández-Rosas et al., 2010).

Si bien la biocompatibilidad fue el primer requisito que debían cumplir los microcódigos, el segundo fue que la tasa de identificación embrionaria fuera elevada. Considerando que la orientación del microcódigo podía ser un parámetro determinante para incrementar la probabilidad de lectura, se diseñaron microcódigos en 3D (tipo A) con la intención de que su lectura fuera más fácil que

la de los microcódigos en 2D (tipos B y C) en cualquier orientación. No obstante, los resultados obtenidos mostraron que las tasas de identificación embrionaria con los microcódigos tipo A eran similares a las logradas con los microcódigos tipo C, y un poco superiores a las obtenidas con los microcódigos tipo B.

Ante la ausencia de diferencias significativas entre las tasas de identificación embrionaria obtenidas con los distintos modelos de microcódigo, el número de combinaciones posibles con cada tipo de microcódigo y la facilidad de su lectura se utilizaron como parámetros de selección. De este modo, los microcódigos 2D (con 8 bits; tipo B y C) permiten un número más elevado de combinaciones que los microcódigos 3D (con 6 bits; tipo A). A su vez, dentro de la categoría de microcódigos 2D, los bits del tipo C pueden ser diseñados en dos diferentes geometrías (tipos C1 y C2) permitiendo doblar el número de posibles combinaciones ofrecidas por el tipo B. Referente al segundo parámetro de selección, se detectó que la lectura bajo el microscopio del microcódigo tipo C resultaba más fácil que la de los otros dos tipos. Finalmente, dadas estas peculiaridades, se consideró el microcódigo tipo C como el más apropiado para su aplicación en el etiquetaje de embriones, y por ello fue el utilizado en los experimentos posteriores para el desarrollo del sistema de etiquetaje directo.

4.2. Desarrollo de un sistema eficiente de vinculación de microcódigos a ovocitos y embriones.

El sistema de etiquetaje directo debería permitir que los microcódigos acompañen al embrión durante todo el proceso *in vitro* para que estos puedan ser identificados y trazados en cada momento que se requiera, y liberarse una vez completado dicho proceso. En especial, es importante que los embriones se liberen de los microcódigos en el momento de su implantación en el útero materno para evitar posibles interferencias. En el primer trabajo de esta tesis se observó que el sistema de vinculación de microcódigos por microinyección en el EPV presentaba una importante limitación: los microcódigos quedaban adheridos a la superficie celular de la mayoría de los embriones después de que

eclosionaran. Dado que la adherencia de los microcódigos no se observó nunca en los estadios de desarrollo previos al blastocisto, puede ser que la reducción o desaparición del EPV durante el proceso de expansión del blastocisto facilite el contacto cercano entre el microcódigo y la superficie celular del trofectodermo, y/o que los cambios en la superficie celular que acompañan a la formación de este linaje (Yamanaka et al., 2006) promuevan la adhesión de los microcódigos. Ante esta limitación, se planteó como una posible alternativa la adhesión de los microcódigos a la superficie externa de la zona pelúcida (ZP) a través de su biofuncionalización con una biomolécula con capacidad de unión a la ZP. Entre las biomoléculas que poseen esta propiedad, se seleccionaron dos lectinas capaces de reconocer sacáridos presentes en la ZP, la del germen de trigo (*Wheat germ agglutinin*, WGA; Avilés et al., 1997) y la fitohemaglutinina-L (*Phytohaemagglutinin-L*, PHA-L; Jiménez-Movilla et al., 2004), así como el anticuerpo anti-mZP2, que reconoce un epítopo específico de la glicoproteína ZP2 de ratón (East et al., 1984). Para determinar cual de estas biomoléculas era la más apropiada para la eventual funcionalización de los microcódigos, se estudiaron las características de la unión a la ZP de todas ellas.

Utilizando microscopía confocal se obtuvieron los patrones de unión a la ZP de las diferentes biomoléculas testadas: externo para la WGA, interno para la PHA-L y en todo el espesor para el anticuerpo anti-mZP2, siendo para todas ellas similares antes y después de la fecundación (después de la reacción de zona). Estos resultados concuerdan con estudios previos realizados con microscopía electrónica de transmisión, que han descrito una distribución uniforme de la unión del anticuerpo anti-mZP2 en todo el espesor de la ZP (Avilés et al., 1997; Jiménez-Movilla et al., 2004) y una unión predominante de la WGA en la región externa de la ZP de ratón (Nicolson et al., 1975).

Mientras que el uso del microscopio confocal permitió determinar la localización preferencial de los puntos de unión de cada biomolécula en el espesor de la ZP, la cuantificación de estos requirió el uso de técnicas de microscopía electrónica, y para este propósito se utilizó el microscopio electrónico de rastreo por emisión de campo. Únicamente se cuantificaron los puntos de unión de la tres moléculas

utilizadas a la superficie externa de la ZP, debido a que estos son los únicos accesibles para las biomoléculas que vayan a ser unidas covalentemente a una micropartícula. Como es sabido, la composición bioquímica de la ZP cambia después de la fecundación o activación partenogenética del ovocito debido a las secreciones de los gránulos corticales que promueven la reacción de zona (Wassarman, 1987). La liberación de proteasas durante este proceso provoca, entre otras modificaciones, la modificación de la ZP2 (Moller et al., 1989). Sin embargo, estas modificaciones no afectaron a la unión del anticuerpo anti-mZP2 a su epítopo, ya que el número de puntos de unión reconocidos en la ZP de ovocitos y embriones fue equivalente. Por otro lado, se observó un descenso de más de la mitad de los puntos de unión de la PHA-L en embriones respecto a ovocitos, pero no de los reconocidos por la WGA, que mantuvo el número de puntos de unión a la ZP después de la fecundación. Estas diferencias pueden deberse a que la complejidad de los sacáridos reconocidos por la PHA-L (Hammarström et al., 1982) es mayor que la de los reconocidos por la WGA (Nagata et al., 1974), siendo probablemente más susceptibles a modificaciones durante la reacción de zona.

A pesar de que el anticuerpo anti-mZP2 y la WGA fueron las biomoléculas con mayor número de puntos de unión por área superficial externa de ZP, ambas resultaron ser tóxicas para el desarrollo embrionario cuando se añadían al medio de cultivo, mientras que la PHA-L no. No obstante, es importante destacar que cuando dichas biomoléculas fueron unidas covalentemente a microesferas comerciales de poliestireno (usadas como modelo), no se observaron efectos tóxicos para el desarrollo embrionario *in vitro.* Esto indica que la unión covalente entre la biomolécula y la microesfera era estable, lo que evitaba la liberación de la biomolécula y su difusión a través de la ZP hasta contactar con las células del embrión.

La biofuncionalización de las microesferas también permitió analizar la capacidad de cada una de las biomoléculas testadas para adherir eficientemente micropartículas a la ZP. A pesar del elevado número de puntos de unión a la ZP observados para el anticuerpo anti-mZP2, las microesferas biofuncionalizadas con esta biomolécula se desprendían más fácilmente de la ZP después de las

manipulaciones iniciales que las biofuncionalizadas con lectinas, indicando que el anticuerpo establecía una unión inicial más débil que la lograda por las lectinas. Como era de esperar por los resultados mencionados anteriormente, las modificaciones bioquímicas de la ZP durante la activación del ovocito no afectaron a la adhesión de las microesferas biofuncionalizadas con WGA o anticuerpo anti-mZP2. Sorprendentemente tampoco afectaron a la adhesión de las microesferas biofuncionalizadas con PHA-L, a pesar de la disminución de más de la mitad de puntos de unión observada después de la reacción de zona. Las diferencias detectadas en la estabilidad de la unión de las microesferas a la ZP entre las diferentes biomoléculas testadas pueden ser atribuidas, en parte, al número de sitios de reconocimiento que presenta cada una de ellas. De este modo, mientras el anticuerpo anti-mZP2 posee dos potenciales sitios de reconocimiento del antígeno, las lectinas tienen cuatro o más sitios de reconocimiento de sacáridos por molécula. Concretamente, la PHA-L tiene cuatro sitios de reconocimiento (Sharon et al., 1972) y la WGA es la única lectina que dispone de múltiples (cuatro) sitios de reconocimiento por monómero, ofreciendo un total de 8 potenciales sitios de unión a carbohidratos (Wright et al., 1996). Estas diferencias, conjuntamente con los resultados de cuantificación de puntos de unión a la superficie externa de la ZP, explicarían la elevada estabilidad de la adhesión a la ZP de las microesferas biofuncionalizadas con WGA durante todo el cultivo *in vitro* en comparación con la PHA-L y con el anticuerpo anti-mZP2.

Por todo lo expuesto, al final del estudio se concluyó que la lectina WGA era, entre las candidatas testadas, la biomolécula más eficiente para biofuncionalizar micropartículas con el objetivo de adherirlas y mantenerlas adheridas a la ZP de ovocitos y embriones de ratón. Además, los sacáridos reconocidos por la WGA son muy abundantes en la ZP de la mayoría de especies de mamíferos (Skutelsky et al., 1994; Habermann et al., 2011), lo que hace suponer que las micropartículas biofuncionalizadas con WGA podrían unirse a la ZP de ovocitos y embriones de diferentes especies de mamíferos.

4.3. Valoración de la inocuidad y efectividad del sistema de etiquetaje directo de ovocitos y embriones.

Una vez seleccionados los microcódigos (tipo C), el sistema de vinculación de estos a los ovocitos/embriones (adhesión a la superficie externa de la ZP) y la biomolécula para biofuncionalizarlos (WGA) se procedió a valorar la efectividad e inocuidad del sistema de etiquetaje directo en embriones de ratón. Desde el inicio el sistema de etiquetaje se diseñó teniendo en cuenta que la lectura del microcódigo debía ser en el futuro mediante un sistema automático de reconocimiento de imágenes. Por lo tanto, para incrementar la probabilidad de que al menos uno de los microcódigos se encontrara en la orientación adecuada para ser leído y el embrión pudiera ser identificado sin su manipulación se adhirieron 10 microcódigos, repartidos homogéneamente por la superficie de la ZP. El proceso de etiquetaje de un embrión utilizando esta metodología requirió simplemente el uso de un microscopio estereoscópico y un sistema de aspiración, evitando el uso de técnicas de micromanipulación para realizar el etiquetaje, imprescindibles para el sistema de vinculación por introducción de microcódigos en el EPV.

Los embriones etiquetados lograron tasas de desarrollo *in vitro* equivalentes a la de los embriones no etiquetados, evidenciando la inocuidad del sistema de etiquetaje y de los microcódigos biofuncionalizados con WGA. Por otro lado las elevadas tasas de retención de microcódigos a lo largo del cultivo *in vitro* de los embriones etiquetados (9,56±0,06 microcódigos/embrión a las 96 h de cultivo) indicaron que el sistema de vinculación desarrollado era efectivo. El desprendimiento de algunos microcódigos en menos del 35% de los embriones durante el cultivo *in vitro* puede ser atribuido al debilitamiento de la unión entre la WGA y la ZP a causa de las modificaciones que la ZP sufre durante el desarrollo embrionario temprano (Vanroose et al., 2000). A pesar de ello, todos los embriones etiquetados retuvieron al menos 7 microcódigos, permitiendo lograr elevadas tasas de identificación (96,5%) sin manipular el embrión en el momento de la lectura de los microcódigos, con la intención de simular una lectura

automática. Sin embargo, como ningún embrión perdió la totalidad de los microcódigos simplemente rotando el embrión se logró una tasa de identificación del 100%.

La aplicación del sistema de vinculación de los microcódigos por adhesión a la ZP permitió que los embriones al eclosionar quedaran libres de los microcódigos, dejándolos adheridos a la ZP vacía. Por ello, es de suponer que la implantación y el desarrollo post-implantacional probablemente ocurra sin ningún contacto entre el embrión y los microcódigos. De hecho, la elevada tasa de descendencia obtenida en este trabajo después de transferir embriones etiquetados a hembras pseudogestantes confirmó que los microcódigos adheridos a la ZP no afectaban adversamente a la eclosión, implantación y desarrollo a término. Debido a su pequeño tamaño fue imposible determinar el destino final de los microcódigos. No obstante, lo más probable es que al degradarse la ZP por la actividad zona-lítica uterina (Lin et al., 2001) los microcódigos sean liberados en el útero materno y expulsados del cuerpo de la madre junto con la eliminación de la decidua en el momento del parto.

Como la criopreservación de embriones es común en los laboratorios de reproducción asistida, se valoró también la eficacia del etiquetaje directo tras someter embriones etiquetados a este proceso. Esta descrito que la criopreservación induce daños físicos en la ZP (Van dern Abbel et al., 2000) así como cambios bioquímicos relacionados con la estructura secundaria de proteínas y carbohidratos (Bogliolo et al., 2012). Por ello, era necesario determinar si la efectividad del sistema de etiquetaje basado en el reconocimiento y unión de la WGA a azucares de las glicoproteínas de la ZP podría verse alterada durante la criopreservación. Los resultados obtenidos en esta serie de experimentos demostraron que la presencia de microcódigos adheridos a la ZP no producía efectos adversos en el desarrollo de los embriones después de su congelación/descongelación. Sin embargo, los embriones descongelados perdieron más microcódigos que los embriones frescos (no congelados) en las primeras horas de cultivo postdescongelación, y consecuentemente el valor medio de retención al final del cultivo y la tasa global de identificación fueron inferiores a

los de embriones no congelados. A pesar de ello, la identificación global seguía siendo superior al 92% y, como todos los embriones descongelados mantenían adheridos al menos 5 microcódigos, con solo rotarlos todos pudieron se identificados.

En conclusión, el método de etiquetaje directo diseñado en este estudio no afectó al desarrollo embrionario *in vitro* ni *in vivo* de los embriones, y permitió una identificación eficiente durante su procesamiento *in vitro*.

4.4. Etiquetaje directo de ovocitos y embriones humanos para su trazabilidad.

Dados los excelentes resultados obtenidos con el sistema de etiquetaje directo por adhesión de microcódigos a la ZP de embriones de ratón, en los siguientes experimentos se testó su aplicación en ovocitos y embriones humanos.

El etiquetaje directo de embriones humanos no afectó al desarrollo *in vitro* de estos. Además, la calidad embrionaria, valorada mediante gradación morfológica en diferentes momentos del desarrollo y por recuento celular de la masa interna, el trofectodermo y el número total de células en blastocistos de día 6, no se vio alterada por el etiquetaje de los embriones. Un análisis más exhaustivo de la competencia embrionaria, realizado mediante la evaluación de parámetros morfocinéticos de los embriones, no mostró tampoco ninguna alteración en el tiempo de ocurrencia ni en la duración de los diferentes eventos analizados, confirmando la inocuidad del sistema de etiquetaje.

Como se había observado previamente en el modelo de ratón, algunos microcódigos se desprendieron de la ZP durante el cultivo *in vitro*, probablemente a causa de la debilitación de las uniones WGA-ZP (Vanroose et al., 2000). A pesar de estas pérdidas, la retención al final del cultivo fue elevada (9,22±0,13 microcódigos/embrión) y equivalente a la observada en los estudios previos en embriones de ratón (9,56±0,06 microcódigos/embrión). Esta elevada retención observada en ambas especies puede ser debida a la alta afinidad de la WGA para unirse a la ZP de ovocitos y embriones de ratón (Skutelsky et al., 1994) y humanos (Maymon et al., 1994; Talevi et al., 1997; Jiménez-Movilla et al., 2004). Como era

de esperar por los valores de retención obtenidos, la identificación global valorada sin manipulación del embrión para simular un proceso automático de lectura fue elevada (96,9%) y equivalente a la obtenida en experimentos previos con embriones de ratón (96,5%).

La efectividad del sistema de etiquetaje también se valoró después de la vitrificación, uno de los procedimientos más comunes en las TRAs humanas. La viabilidad de los embriones etiquetados no se vio alterada después de la vitrificación, pero si que se observó una disminución significativa del número de microcódigos unidos a la ZP (antes: 9,35±0,13 y después: 7,33±0,44 de la vitrificación), posiblemente debido a los daños físicos (Van den Abbel et al., 2000) y químicos (Bogliolo L et al., 2012) que la ZP experimenta durante procesos de criopreservación como la vitrificación. Este descenso del número medio de microcódigos adheridos por embrión afectó directamente a la identificación global que, pese a ser elevada, descendió hasta el 88,9% mientras que en embriones no vitrificados se situó en el 96,9%. A pesar de ello, como los embriones sometidos a vitrificación mantenían al menos 7 microcódigos, todos ellos podían ser identificados con simplemente rotarlos.

Finalmente, el sistema de etiquetaje se aplicó a ovocitos humanos y su efectividad se valoró durante una simulación de ICSI. La presencia de los microcódigos adheridos a la ZP no interfirió en el procedimiento de micromanipulación y además todos los microcódigos permanecieron unidos a la ZP. Tampoco se observó desprendimiento de microcódigos después de la secreción de los gránulos corticales en los ovocitos activados partenogenéticamente, demostrando que después de la reacción de zona se mantiene un número suficiente de puntos de unión de WGA para conservar adherido el microcódigo a la ZP, como ya habíamos observado anteriormente en ratón.

A pesar de que el etiquetaje directo es posible para ovocitos que vayan a ser fecundados mediante ICSI, el sistema de etiquetaje directo desarrollado no permite el etiquetaje de ovocitos destinados a FIV convencional, ya que para este procedimiento es necesario mantener las células del cumulus adheridas a la ZP. Como el etiquetaje directo desarrollado requiere de la previa denudación del

ovocito para permitir la unión de la WGA a la ZP, los ovocitos fecundados por FIV convencional no podrían ser etiquetados hasta después de la fecundación. Por la misma razón, otra limitación del sistema es el etiquetaje de ovocitos que requieren de una maduración *in vitro* ya que las células del cumulus han de mantenerse adheridas a la ZP.

En conclusión, la aplicación del sistema de etiquetaje directo en ovocitos y embriones humanos es un método eficiente y económico para su identificación durante la mayoría de los procesos a los que estas muestras son sometidas durante su paso por el laboratorio de fecundación *in vitro*.

A pesar de las limitaciones del sistema de etiquetaje directo su implementación en centros de reproducción asistida podría cubrir la mayoría de los puntos críticos de preservación de la trazabilidad de muestras reproductivas que actualmente están al descubierto, principalmente los continuos cambios de recipiente. Además este sistema es compatible con los sistemas de etiquetaje de recipientes ya existentes (marcaje manual y etiquetas electrónicas), y su uso conjunto ofrecería un grado más de seguridad en la trazabilidad de estas muestras, disminuyendo el riesgo de *mix-ups*.

4.5. Etiquetaje directo de embriones bovinos para su cultivo en grupo.

La trazabilidad de embriones bovinos durante el cultivo *in vitro* es importante para algunas aplicaciones, por ejemplo en los procesos de producción *in vitro* de embriones de ejemplares de alto valor genético. La utilización del sistema de etiquetaje directo por adhesión de microcódigos a la ZP de estos embriones puede permitir el cultivo colectivo de embriones de diferentes orígenes preservando su identificación durante todo el cultivo. Cabe señalar que en los experimentos llevados a cabo en esta tesis los embriones utilizados procedían de ovocitos obtenidos de ovarios de hembras de matadero. Estos embriones, de fácil y económica producción, se utilizaron como modelo para testar la aplicación del sistema de etiquetaje directo para el cultivo colectivo de embriones con diferentes orígenes producidos a partir de ovocitos obtenidos mediante OPU.

El etiquetaje directo de embriones bovinos con microcódigos biofuncionalizados con WGA no afectó a su desarrollo ni a la calidad de los blastocistos producidos. Cada embrión se etiquetó con ocho microcódigos con el objetivo de que al menos uno de ellos permaneciera adherido hasta el estadio de blastocisto, manteniendo así identificado al embrión durante todo el cultivo. El desprendimiento de microcódigos fue más elevado en este estudio, con embriones bovinos, que en los anteriores, con embriones de ratón y humanos. Esto sugiere que las ZPs de ratón y de humano quizás posean más puntos de unión en la superficie externa reconocibles por la WGA que la ZP de bovino. Esto concuerda con observaciones realizadas por otros investigadores en que indican que la unión de la WGA se localiza predominante en la zona externa de la ZP de ratón (Nicolson et al., 1975) y de humano, mientras que en la ZP de bovino lo hace preferentemente localizado en la zona interna (Habermann et al., 2011). Una vez los microcódigos se desprendían, nunca volvían a adherirse a la ZP del mismo ni de otro embrión, permaneciendo todos ellos en el fondo del soporte de cultivo. A pesar de las pérdidas, todos los embriones conservaron como mínimo un microcódigo adherido a su ZP hasta el estadio de blastocisto, lo que era suficiente para lograr su identificación. Aunque el etiquetaje permite la identificación del embrión en cualquier momento del cultivo, en este estudio sólo se realizó el análisis de la identificación al final del cultivo ya que en bovino el estadio más óptimo para la transferencia o criopreservación es el de blastocisto (Lamb, 2005; Morató et al., 2010) y el cultivo se realiza en un medio global. La identificación se realizó fácil y rápidamente (20 segundos/embrión) mediante reconocimiento visual de la codificación del microcódigo bajo un microscopio invertido (200x magnificación). Como el 100% de los embriones mantenían como mínimo un microcódigo, el 100% de los blastocistos pudo ser identificado simplemente rotando el embrión hasta que un microcódigo quedara correctamente orientado para su lectura.

Después de la vitrificación, la presencia de microcódigos no afectó la reexpansión de los blastocitos etiquetados. Esto, conjuntamente con el recuento celular en el estadio de blastocisto, mostró que la recuperación y la calidad de los embriones

etiquetados era equivalente a la de los embriones no etiquetados después de la vitrificación.

La aplicación del etiquetaje directo en embriones bovinos ofrece varias ventajas respecto a los demás sistemas desarrollados con el fin de mantener la trazabilidad embrionaria en un cultivo en grupo. Una de ellas es que este sistema no requiere la modificación física del soporte de cultivo como los sistemas de micropocillos y Cell-Tak, que son de construcción laboriosa (Matoba et al., 2010). Otra es que los embriones etiquetados se mantienen identificados durante todo el periodo de cultivo incluso después de cambios de medio o manipulaciones, ya que la etiqueta viaja con ellos, mientras que utilizando los sistemas actualmente disponibles estas manipulaciones son puntos críticos para preservar la trazabilidad. Finalmente, como la identificación del embrión no está relacionada con una posición concreta en el soporte de cultivo, el embrión permanece identificado incluso después de un movimiento brusco de la placa de cultivo, cuando con los sistemas disponibles la identificación se perdería (Goovaerts et al., 2010; Somfai et al., 2010).

En conclusión, el sistema de etiquetaje directo de embriones bovinos es efectivo, inocuo y permite el cultivo de embriones procedentes de diferentes orígenes compartiendo el mismo medio de cultivo, manteniendo así los beneficios del cultivo colectivo en un soporte tradicional. Además, en contraste con los otros sistemas, el etiquetaje con microcódigos biofuncionalizados con WGA podría combinarse con plataformas de cultivo dinámico, novedosos sistemas de cultivo que han mostrado resultados prometedores en la producción *in vitro* de embriones (Smith et al., 2012). Este nuevo sistema de etiquetaje e identificación podría mejorar la eficiencia de los procesos de OPU/FIV y facilitar la producción de embriones de animales vivos de alto valor genético.

5. CONCLUSIONES

1) Entre los microcódigos testados, el tipo C es el más eficiente para etiquetar e identificar ovocitos y embriones de mamífero por su fácil lectura y número de posibles combinaciones.
2) El sistema más eficiente para lograr la vinculación de los microcódigos a ovocitos y embriones es su adhesión a la zona pelúcida una vez biofuncionalizados con la lectina WGA.
3) El etiquetaje directo de embriones de ratón con 10 microcódigos biofuncionalizados con WGA adheridos y repartidos homogéneamente en la superficie de la ZP no afecta al desarrollo *in vitro* (90% de blastocistos) ni *in vivo* (74,6% de nacimientos), se mantiene hasta el final del procesamiento de la muestra (9,56±0,06 microcódigos/embrión) y permite la identificación en el 96,5% de los casos sin la manipulación del embrión.
4) El etiquetaje directo de embriones humanos con 10 microcódigos no afecta al desarrollo *in vitro* (46,7% de blastocistos) ni a la calidad embrionaria, se mantiene hasta el final del procesamiento de la muestra (9,22±0,13 microcódigos/embrión) y permite la identificación en el 96,9% de los casos sin la manipulación del embrión.
5) La aplicación del sistema de etiquetaje directo en ovocitos y embriones humanos es un método eficiente para su identificación durante la mayoría de los procesos a los que son sometidos en el laboratorio de fecundación *in vitro* (cultivo, criopreservación y ICSI), y es por lo tanto una herramienta potencialmente útil para prevenir *mix-ups*.
6) El etiquetaje directo de embriones bovinos con 8 microcódigos no afecta al desarrollo *in vitro* (34,8% de blastocistos) ni a la calidad embrionaria, se mantiene hasta el final del procesamiento de la muestra (3,63±0,37 microcódigos/embrión) y permite la identificación manual en el 100% de los casos.
7) La aplicación del sistema de etiquetaje directo en embriones bovinos permite el cultivo de embriones procedentes de diferentes orígenes

compartiendo el mismo medio de cultivo, manteniendo así los beneficios del cultivo colectivo en un soporte tradicional.

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7. ANEXO

Otras publicaciones relacionadas con esta Tesis Doctoral:

Penon O, **Novo S**, Duran S, Oncins G, Siapkas D, Errachid A, Barrios L, Nogues C, Duch M, Plaza JA, Perez-Garcia L (2013) Optimized immobilization of lectins using self-assembled monolayers on polysilicon encoded materials for cell tagging. Nanotechnology (bajo revisión).

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“You must be shapeless, formless, like water. When you pour water in a cup, it becomes the cup. When you pour water in a bottle, it becomes the bottle. When you pour water in a teapot, it becomes the teapot. Water can drip and it can crash. Become like water my friend.”

Bruce Lee