CAMBRIDGE JOURNALS **Proof Delivery Form**

Zygote

Date of delivery:

Journal and vol/article ref: ZYG 1200008

Number of pages (not including this page): 9

This proof is sent to you on behalf of Cambridge University Press. Please print out the file and check the proofs carefully. Make any corrections necessary on a hardcopy and answer queries on each page of the proofs.

Please return the **marked proof** within **2** days of receipt to:

Nicki Marshall
Cambridge University Press
The Edinburgh Building,
Shaftesbury Road
Cambridge CB2 8RU, UK **Email: nmarshall@cambridge.org**

Authors are strongly advised to read these proofs thoroughly because any errors missed may appear in the final published paper. This will be your ONLY chance to correct your proof. Once published, either online or in print, no further changes can be made.

To avoid delay from overseas, please send the proof by airmail or courier.

If you have **no corrections** to make, please email **nmarshall@cambridge.org** to save having to return your paper proof. If corrections are light, you can also send them by email, quoting both page and line number.

- The proof is sent to you for correction of typographical errors only. Revision of the substance of the text is not permitted, unless discussed with the editor of the journal. Only **one** set of corrections are permitted.
- Please answer carefully any author queries.
- Corrections which do NOT follow journal style will not be accepted.
- A new copy of a figure must be provided if correction of anything other than a typographical error introduced by the typesetter is required.

- If you have problems with the file please contact **nmarshall@cambridge.org**

Please note that this pdf is for proof checking purposes only. It should not be distributed to third parties and may not represent the final published version.

Important: you must return any forms included with your proof. We cannot publish your article if you have not returned your signed copyright form.

NOTE - for further information about **Journals Production** please consult our **FAQs** at http://journals.cambridge.org/production_faqs

Author queries:

Typesetter queries:

Non-printed material:

Offprint order form

CAMBRIDGE UNIVERSITY PRESS

PLEASE COMPLETE AND RETURN THIS FORM. WE WILL BE UNABLE TO SEND OFFPRINTS UNLESS A RETURN ADDRESS AND ARTICLE DETAILS ARE PROVIDED.

VAT REG NO. GB 823 8476 09

Zygote (ZYG)

Volume: ____ *no:* ____

Offprints

To order offprints, please complete this form and send it to **the publisher (address below)**. Please give the address to which your offprints should be sent. They will be despatched by surface mail within one month of publication. For an article by **more than one author this form is sent to you as the first named author.**

Number of offprints required: ____

Email: ----------

Offprints to be sent to (print in BLOCK CAPITALS): ----------

---

Post/Zip Code: ----------

Telephone: ---------- *Date (dd/mm/yy):* ____ / ____ / ____

Author(s):

Article Title: ----------

---

*All enquiries about offprints should be addressed to **the Publisher**: Journals Production Department, Cambridge University Press, University Printing House, Shaftesbury Road, Cambridge CB2 8BS, UK.*

Charges for offprints (excluding VAT) *Please circle the appropriate charge:*

Number of copies	25	50	100	150	200	per 50 extra
1-4 pages	£68	£109	£174	£239	£309	£68
5-8 pages	£109	£163	£239	£321	£399	£109
9-16 pages	£120	£181	£285	£381	£494	£120
17-24 pages	£131	£201	£331	£451	£599	£131
Each Additional 1-8 pages	£20	£31	£50	£70	£104	£20

Methods of payment

If you live in Belgium, France, Germany, Ireland, Italy, Portugal, Spain or Sweden and are not registered for VAT we are required to charge VAT at the rate applicable in your country of residence. If you live in any other country in the EU and are not registered for VAT you will be charged VAT at the UK rate.
If registered, please quote your VAT number, or the VAT number of any agency paying on your behalf if it is registered. *VAT Number:* ----------

Payment **must** be included with your order, please tick which method you are using:

☐ Cheques should be made out to Cambridge University Press.

☐ Payment by someone else. Please enclose the official order when returning this form and ensure that when the order is sent it mentions the name of the journal and the article title.

☐ Payment may be made by any credit card bearing the Interbank Symbol.

Card Number: | | | | | | | | | | | | | | | | |

Expiry Date (mm/yy): ____ / ____ *Card Verification Number:* | | | |

The card verification number is a 3 digit number printed on the **back** of your **Visa** or **Master card**, it appears after and to the right of your card number. For **American Express** the verification number is 4 digits, and printed on the **front** of your card, after and to the right of your card number.

Signature of card holder: ---------- *Amount (Including VAT if appropriate):* £ ----------

Please advise if address registered with card company is different from above

Journal transfer of copyright

CAMBRIDGE UNIVERSITY PRESS

Please read the notes overleaf and then complete, sign, and return this form to **Journals Production, Cambridge University Press, University Printing House, Shaftesbury Road, Cambridge, CB2 8BS, UK** as soon as possible. Please complete both **Sections A and B**.

Zygote

In consideration of the publication in **Zygote**

of the contribution entitled: .....

.....

by (all authors' names): .....

.....

Section A – Assignment of Copyright (fill in either part 1 or 2 or 3)

1 To be filled in if copyright belongs to you

Transfer of copyright

I/we hereby assign to Cambridge University Press, full copyright in all forms and media in the said contribution, including in any supplementary materials that I/we may author in support of the online version.

I/we hereby assert my/our moral rights in accordance with the UK Copyright Designs and Patents Act (1988).

Signed (tick one) □ the sole author(s)

□ one author authorised to execute this transfer on behalf of all the authors of the above article

Name (block letters) .....

Institution/Company .....

Signature: ..... Date: .....

(Additional authors should provide this information on a separate sheet.)

2 To be filled in if copyright does not belong to you

a Name and address of copyright holder .....

.....

.....

b The copyright holder hereby grants to Cambridge University Press, the non-exclusive right to publish the contribution in the Journal including any supplementary materials that support the online version and to deal with requests from third parties.

(Signature of copyright holder or authorised agent).....

3 US Government exemption

I/we certify that the paper above was written in the course of employment by the United States Government so that no copyright exists.

Signature:..... Name (Block letters): .....

Section B – Warranty and disclosure of conflict of interest

I/we warrant that I am/we are the sole owner or co-owners of the contribution and have full power to make this agreement, and that the contribution contains nothing that is in any way an infringement of any existing copyright or licence, or duty of confidentiality, or duty to respect privacy, or any other right of any person or party whatsoever and contains nothing libellous or unlawful; and that all statements purporting to be facts are true and that any recipe, formula, instruction or equivalent published in the Journal will not, if followed accurately, cause any injury or damage to the user.

I/we further warrant that permission for all appropriate uses has been obtained from the copyright holder for any material not in my/our copyright including any audio and video material, that the appropriate acknowledgement has been made to the original source, and that in the case of audio or video material appropriate releases have been obtained from persons whose voices or likenesses are represented therein. I/we attach copies of all permission and release correspondence.

I indemnify and keep Cambridge University Press indemnified against any loss, injury or damage (including any legal costs and disbursements paid by them to compromise or settle any claim) occasioned to them in consequence of any breach of these warranties.

Name (block letters)……..

Signature …… Date ……

(one author authorised to execute this warranty statement above and conflict of interest statement below on behalf of all the authors of the above article)

Please disclose any potential **conflict of interest** pertaining to your contribution or the Journal; or write 'NONE' to indicate you declare no such conflict of interest exists. A conflict of interest might exist if you have a competing interest (real or apparent) that could be considered or viewed as exerting an undue influence on you or your contribution. Examples could include financial, institutional or collaborative relationships. The Journal's editor(s) shall contact you if any disclosed conflict of interest may affect publication of your contribution in the Journal.

Potential conflict of interest ……

Notes for contributors

1 The Journal's policy is to acquire copyright in all contributions. There are two reasons for this:
(a) ownership of copyright by one central organisation tends to ensure maximum international protection against unauthorised use; *(b)* it also ensures that requests by third parties to reprint or reproduce a contribution, or part of it, are handled efficiently and in accordance with a general policy that is sensitive both to any relevant changes in international copyright legislation and to the general desirability of encouraging the dissemination of knowledge.

2 Two 'moral rights' were conferred on authors by the UK Copyright Act in 1988. In the UK an author's 'right of paternity', the right to be properly credited whenever the work is published (or performed or broadcast), requires that this right is asserted in writing.

3 Notwithstanding the assignment of copyright in their contribution, all contributors retain the following **non-transferable** rights:

- The right (subject to appropriate permission having been cleared for any third-party material) to post *either* their own version of their contribution as submitted to the journal (prior to revision arising from peer review and prior to editorial input by Cambridge University Press) *or* their own final version of their contribution as accepted for publication (subsequent to revision arising from peer review but still prior to editorial input by Cambridge University Press) on their **personal or departmental web page**, or in the **Institutional Repository** of the institution in which they worked at the time the paper was first submitted, or (for appropriate journals) in PubMedCentral or UK PubMedCentral, provided the posting is accompanied by a prominent statement that the paper has been accepted for publication and will appear in a revised form, subsequent to peer review and/or editorial input by Cambridge University Press, in **Zygote** published by Cambridge University Press, together with a copyright notice in the name of the copyright holder (Cambridge University Press or the sponsoring Society, as appropriate). On publication the full bibliographical details of the paper (volume: issue number (date), page numbers) must be inserted after the journal title, along with a link to the Cambridge website address for the journal. Inclusion of this version of the paper in Institutional Repositories outside of the institution in which the contributor worked at the time the paper was first submitted will be subject to the additional permission of Cambridge University Press (not to be unreasonably withheld).
- The right (subject to appropriate permission having been cleared for any third-party material) to post the definitive version of the contribution as published at Cambridge Journals Online (in PDF or HTML form) on their **personal or departmental web page**, no sooner than upon its appearance at Cambridge Journals Online, subject to file availability and provided the posting includes a prominent statement of the full bibliographical details, a copyright notice in the name of the copyright holder (Cambridge University Press or the sponsoring Society, as appropriate), and a link to the online edition of the journal at Cambridge Journals Online.
- The right (subject to appropriate permission having been cleared for any third-party material) to post the definitive version of the contribution as published at Cambridge Journals Online (in PDF or HTML form) in the **Institutional Repository** of the institution in which they worked at the time the paper was first submitted, or (for appropriate journals) in PubMedCentral or UK PubMedCentral, no sooner than **one year** after first publication of the paper in the journal, subject to file availability and provided the posting includes a prominent statement of the full bibliographical details, a copyright notice in the name of the copyright holder (Cambridge University Press or the sponsoring Society, as appropriate), and a link to the online edition of the journal at Cambridge Journals Online. Inclusion of this definitive version after one year in Institutional Repositories outside of the institution in which the contributor worked at the time the paper was first submitted will be subject to the additional permission of Cambridge University Press (not to be unreasonably withheld).
- The right to post an abstract of the contribution (for appropriate journals) on the Social Science Research Network (SSRN), provided the abstract is accompanied by a prominent statement that the full contribution appears in **Zygote** published by Cambridge University Press, together with full bibliographical details, a copyright notice in the name of the journal's copyright holder (Cambridge University Press or the sponsoring Society, as appropriate), and a link to the online edition of the journal at Cambridge Journals Online.
- The right to make hard copies of the contribution or an adapted version for their own purposes, including the right to make multiple copies for course use by their students, provided no sale is involved.
- The right to reproduce the paper or an adapted version of it in any volume of which they are editor or author. Permission will automatically be given to the publisher of such a volume, subject to normal acknowledgement.

4 Cambridge University Press co-operates in various licensing schemes that allow material to be photocopied within agreed restraints (e.g. the CCC in the USA and the CLA in the UK). Any proceeds received from such licenses, together with any proceeds from sales of subsidiary rights in the Journal, directly support its continuing publication.

5 It is understood that in some cases copyright will be held by the contributor's employer. If so, Cambridge University Press requires non-exclusive permission to deal with requests from third parties.

6 Permission to include material not in your copyright
If your contribution includes textual or illustrative material not in your copyright and not covered by fair use / fair dealing, permission must be obtained from the relevant copyright owner (usually the publisher or via the publisher) for the non-exclusive right to reproduce the material worldwide in all forms and media, including electronic publication. The relevant permission correspondence should be attached to this form.

7 Cambridge University Press shall provide the first named author with offprints or/ and a final PDF file of their article, as agreed with sponsoring Society.

If you are in doubt about whether or not permission is required, please consult the Permissions Manager, Cambridge University Press, The Edinburgh Building, Shaftesbury Road, Cambridge CB2 8RU, UK. Fax: +44 (0)1223 315052. Email: lnicol@cambridge.org.

The information provided on this form will be held in perpetuity for record purposes. The name(s) and address(es) of the author(s) of the contribution may be reproduced in the journal and provided to print and online indexing and abstracting services and bibliographic databases

Please make a duplicate of this form for your own records

Zygote **00** (????), pp. 1–9. © Cambridge University Press 2012
doi:10.1017/S0967199412000081

Oocyte genome cloning used in biparental bovine embryo reconstruction

Gabriel Vichera[2], *Ramiro Olivera*[2] *and Daniel Salamone*[1]
Laboratorio de Biotecnología Animal, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

Date submitted: 01.02.2011. Date accepted: 31.03.2011

Summary

Oocyte genome cloning is a method by which haploid maternal embryos are obtained in such a way that parthenogenetic haploid blastomeres from these embryos can be considered as a clone of the original gamete. Our objective was to generate oocyte genome replicates and use them to reconstruct biparental embryos by fusion with haploid male hemizygotes. Furthermore, we generated biparental homogeneous transgene-expressing embryos using parthenogenetic haploid blastomeres that expressed a transgene (EGFP). In the first experiment, parthenogenetic haploid embryos were generated by incubation of oocytes in ionomycin and 6-dimethylaminopurine (DMAP) with a 3 h interval to permit their second polar body extrusion. The cleavage rate was 87.3%. To generate transgene-expressing blastomeres, activated oocytes were injected with pCX–EGFP–liposome complexes 3 h post ionomycin exposure, resulting in a cleavage rate of 84.4%. In the second experiment, haploid parthenogenetic blastomeres that were positive or negative for EGFP expression were used to reconstruct biparental embryos. Cleavage and blastocyst rates for the reconstructed embryos were 78.4% and 61.1% and 10.8% and 8.4%, using EGFP-positive or -negative blastomeres, respectively ($P < 0.05$). All of the reconstructed embryos showed EGFP expression, with 96.6% of them showing homogenic expression. Oct-4 expression in the reconstructed blastocysts displayed a similar pattern as IVF-blastocyst controls. In conclusion, our results proved that it is possible to use oocyte genome replicates to reconstruct biparental bovine embryos and that this technique is efficient to generate homogeneous transgene-expressing embryos.

Keywords: Bovine, Cloning, Gamete, Oocyte, Transgenic

Introduction

Oocyte genome cloning is a method by which haploid parthenogenetic embryos can be obtained in such a way that blastomeres from these embryos can be considered as a clone of the original gamete (Surani *et al.*, 1986; Escribá *et al.*, 2001). Parthenogenetic activation can be stimulated in several mammalian species, including humans (Revazova *et al.*, 2007), by a wide variety of artificial chemical and physical stimuli that induce oocyte activation. These include electrical stimulation, and treatment with ethanol, thimerosal, strontium, ionomycin (Io) or calcium ionophore (Collas *et al.*, 1993; Machaty *et al.*, 1997; Loi *et al.*, 1998; Rho *et al.*, 1998; Liu *et al.*, 2002; Yi *et al.*, 2005; Bhak *et al.*, 2006; Méo *et al.*, 2007). In the bovine, Io in combination with 6-dimethylaminopurine (DMAP; non-specific kinase inhibitors) has been shown to be particularly effective in inducing oocyte activation and subsequent embryo development (Susko-Parrish *et al.*, 1994; Wells *et al.*, 1999).

Ionomycin in combination with DMAP (Io + DMAP) was used to activate cytoplasts during nuclear transfer that produced a cloned bovine, demonstrating the suitability of this treatment in promoting full-term development (Cibelli *et al.*, 1998; Salamone *et al.*, 2006). However, if oocytes activated by Io + DMAP are treated with a 3-h interval between Io activation and DMAP (Io + 3h + DMAP), allowing time for the

[1]All correspondence to: D. Salamone. Laboratorio de Biotecnología Animal, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417 Buenos Aires, Argentina. Tel: + 54 11 4524 8000/8196. Fax: + 54 11 4514 8737. E-mail: salamone@agro.uba.ar
[2]Laboratorio de Biotecnología Animal, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417 Buenos Aires, Argentina.

extrusion of the second polar body (2PB), a specifically haploid parthenote is induced (Susko-Parrish *et al.*, 1994; Vichera *et al.*, 2009). If a 3-h interval between Io and DMAP treatments is absent, the 2PB is not extruded in most activated oocytes, resulting in the formation of diploid parthenotes, (Susko-Parrish *et al.*, 1994; Wells *et al.*, 1999; Cibelli *et al.*, 1998; Salamone *et al.*, 2006; Vichera *et al.*, 2009).

Previous reports on haploid parthenogenetic activation have indicated that no difference in cleavage ability was found between haploid and diploid parthenogenetic embryos; however only some of the haploid parthenogenetic embryos developed to the blastocyst stage (Surani *et al.*, 1986; Escribá *et al.*, 1999, 2000; Lagutina *et al.*, 2004). Parthenogenetic embryos are unable to develop to term due to the imprinting process that has an essential role during embryogenesis (MacGrath *et al.*, 1983, 1984; Surani *et al.*, 1984, 1986). Therefore, to obtain viable offspring from haploid parthenogenetic embryos, every single blastomere from these embryos should be combined with the male counterpart (haploid male hemizygotes) in order to restore the normal heteroparental condition. Haploid male hemizygous eggs can be produced by removing the female pronucleus from fertilized eggs (Barton *et al.*, 1984; McGrath and Solter 1984; Surani *et al.*, 1984; Kaufman *et al.*, 1989; Latham and Solter 1991; Hagemann *et al.*, 1992), fertilization of enucleated oocytes (McGrath and Solter 1984; Surani *et al.*, 1984; Kaufman *et al.*, 1989; Latham and Solter 1991; Hagemann *et al.*, 1992; Obata *et al.*, 2000) and injection of spermatozoa into oocytes followed by maternal chromosomes removal (Miki *et al.*, 2009).

In this work, we compared the developmental ability of haploid and diploid parthenogenetic bovine embryos. Once obtained, blastomeres of haploid parthenogenetic embryos were used, as female genome donors, to reconstruct biparental embryos by fusion with haploid male hemizygotes. In addition, we generated homogeneous transgene-expressing embryos by fusing parthenogenetic haploid blastomeres that expressed a transgene, with haploid male hemizygotes.

Materials and methods

Reagents

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA), except when otherwise indicated.

Oocyte collection and in vitro maturation

Ovaries were collected at a slaughterhouse and transported to the laboratory. Cumulus–oocyte complexes were aspirated from follicles with a diameter of 2 to 8 mm into Dulbecco's phosphate-buffered saline (PBS; GIBCO BRL, Grand Island, NY, USA) that contained 10% fetal bovine serum (FBS; GIBCO BRL) and 2% antibiotic–antimycotic (ATB; GIBCO BRL). Follicular oocytes covered by at least three layers of granulosa cells and with an evenly granulated cytoplasm were selected for *in vitro* maturation. The maturation medium was bicarbonate-buffered TCM-199 (GIBCO BRL) that contains 2 mM glutamine, 10% FBS, 2 μg/ml follicle-stimulating hormone (Follitropin®, Bioniche, Belleville, Ontario, Canada), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768) and 2% ATB. Oocytes were incubated in 100-μl droplets of medium covered with mineral oil (M8410), in 32 mm Petri dishes. *In vitro* maturation conditions were 6.5% CO_2 in humidified air at 39°C for 22 h.

Production of parthenogenetic haploid and diploid embryos

Matured oocytes vortexed for 2 min in hyaluronidase (1 mg/ml in DPBS) to remove cumulus cells, and washed three times in TALP-H (Bavister *et al.*, 1977). Metaphase II (MII) oocytes were identified by first polar body visualization and immediately used for parthenogenetic activation or micromanipulation techniques. Parthenogenetic haploid activation consisted of: (1) incubation with 5 μM Io (Invitrogen, California, USA) for 4 min; (2) incubation with 5 μM Io for 4 min, then in synthetic oviductal fluid (SOF) for 3 h to permit extrusion of the second polar body (2PB), and finally placed in 1.9 mM 6-DMAP in SOF for 3 h. For parthenogenetic diploid activation, oocytes were placed in 5 μM Io for 4 min, followed by 1.9 mM 6-DMAP in SOF for 3 h.

DNA construction

The plasmid used was pCX–EGFP that contains enhanced green fluorescent protein gene (*egfp*) under the control of chimeric cytomegalovirus-IE–chicken β-actin enhancer–promoter (Ikawa *et al.*, 1995).

Liposome–DNA coincubation

For the injection experiments, 1 μl of 4 μg/ml DNA in combination with 3 μl of commercial liposome (Fugene; Boehringer-Manheim, Germany) were coincubated for 15 min. The liposome–DNA mixture was diluted to half concentration with 10% polyvinylpyrrolidone (PVP; Irvine Scientific, Santa Ana, CA, USA), resulting in a final DNA concentration of 0.5 μg/ml.

Intracytoplasmic injection of DNA–liposome complexes

After 3 h of haploid parthenogenetic activation, the ooplasm of the activated oocytes was injected with approximately 2 pl of DNA–liposome/PVP mixture, using an injection capillary (0.7 μm in diameter) attached to a Narishige hydraulic micromanipulator (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA).

Haploid male hemizygotes production

Haploid male hemizygotes were generated by fertilization of enucleated oocytes. Enucleation was performed as follows: oocytes were held and manipulated in TALP-H supplemented with 3 mg/ml bovine serum albumin (BSA). Denuded MII oocytes were enucleated mechanically using micromanipulators mounted on a Nikon Eclipse E-300 microscope and 20 μm diameter pipettes. Metaphase chromosomes were visualized under UV (< 10 s) after staining with 5 μg/ml Hoechst 33342 for 10 min. Fertilization of enucleated oocytes was performed as follows: bovine frozen semen was thawed in a 37°C water bath for 30 s. Spermatozoa were centrifuged twice (490 *g*, 5 min) and resuspended in Brackett–Oliphant medium (BO) supplemented with 5 mM caffeine and 20 IU/ml heparin. Spermatozoa were diluted to half concentration with BO that contained 10 mg/ml fatty acid-free BSA (A6003), resulting in a final sperm concentration of 15×10^6/ml. Spermatozoa were coincubated with enucleated oocytes in 100-μl droplets, for 3 h at 39°C in a humidified atmosphere of 5% CO_2 in air. After this incubation, hemizygotes were immediately used for biparental embryo reconstruction.

Karyotype analysis

Embryos were cultured in SOF supplemented with 0.05 g/ml demecolcine (D1925) for 6 h at 72 h after IVF (in vitro fertilization) and parthenogenetic activation. Embryos were then exposed to a hypotonic 0.8% sodium citrate solution for 10 min at 37°C. Subsequently, embryos were placed on a clean glass slide and treated with a drop of methanol–acetic acid solution (3:1). Slides were dried and then stained with 5% Giemsa solution (Invitrogen) for 10 min. Chromosome spreads were evaluated at ×400 magnification.

Biparental bovine embryo reconstruction by parthenogenetic haploid blastomere fusion

Parthenogenetic haploid embryos (2–16 cells) that expressed EGFP, or not, were treated with 1.5 mg/ml pronase (Sigma protease) dissolved in TALP-H to remove the zona pellucida (ZP). Gentle pipetting was applied to disaggregate blastomeres from these embryos. Parthenogenetic blastomeres that expressed EGFP were then selected under blue light using an excitation filter at 488 nm and an emission filter at 530 nm. Parthenogenetic blastomeres were fused with haploid male hemizygotes as described below. Haploid male hemizygotes were incubated in 1.5 mg/ml pronase for 5–10 min on a warm plate to remove the ZP. ZP-free haploid male hemizygotes were then transferred individually to a drop of 1 mg/ml phytohemaglutinin dissolved in TCM-199 without serum for a few seconds. Following this step, they were dropped quickly over a single parthenogenetic haploid blastomere resting on the bottom of a 100 μl TALP-H drop. Following attachment, the ZP-free haploid male hemizygote/parthenogenetic haploid blastomere pair was picked up, transferred to fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 1 mg/ml PVA), for 2–3 min and then to a fusion chamber (BTX Instrument Division; Harvard Apparatus, Holliston, MA, USA) that contained 2 ml of warm fusion medium. Fusion (Fig. 1) was performed with a double direct current (dc) pulse of 65 V, each pulse was for 30 ms, 0.1 s apart. The biparental reconstructed zygotes were then removed carefully and placed in culture.

In vitro culture

Parthenogenetic, IVF and reconstructed embryos were cultured in SOF medium in a system similar to the well of the well (WOW) method (Vajta *et al.*, 2000), whereby microwells were produced using a heated glass capillary slightly pressed to the bottom of a Petri dish and covered with a 100-μl microdrop of culture medium (16 WOW each microdrop, one embryo each WOW). Culture conditions were a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in air, at 39°C. The medium was supplemented with 10% FBS on day 5 during embryo culture. Cleavage was evaluated on day 2 and blastocysts (Fig. 2) on day 7, post fusion.

Determination of EGFP fluorescence in embryos

Embryos were briefly exposed to blue light during *in vitro* culture, using an excitation filter at 488 nm and an emission filter at 530 nm, to determine EGFP expression at different stages of development. Embryos were analyzed on days 3 and 7, after parthenogenetic activation or biparental embryo reconstruction.

Determination of blastocyst cell number

Embryos were stained in TCM-199 containing 1 mg/ml Hoechst 33342 (B2261), for 2 min and

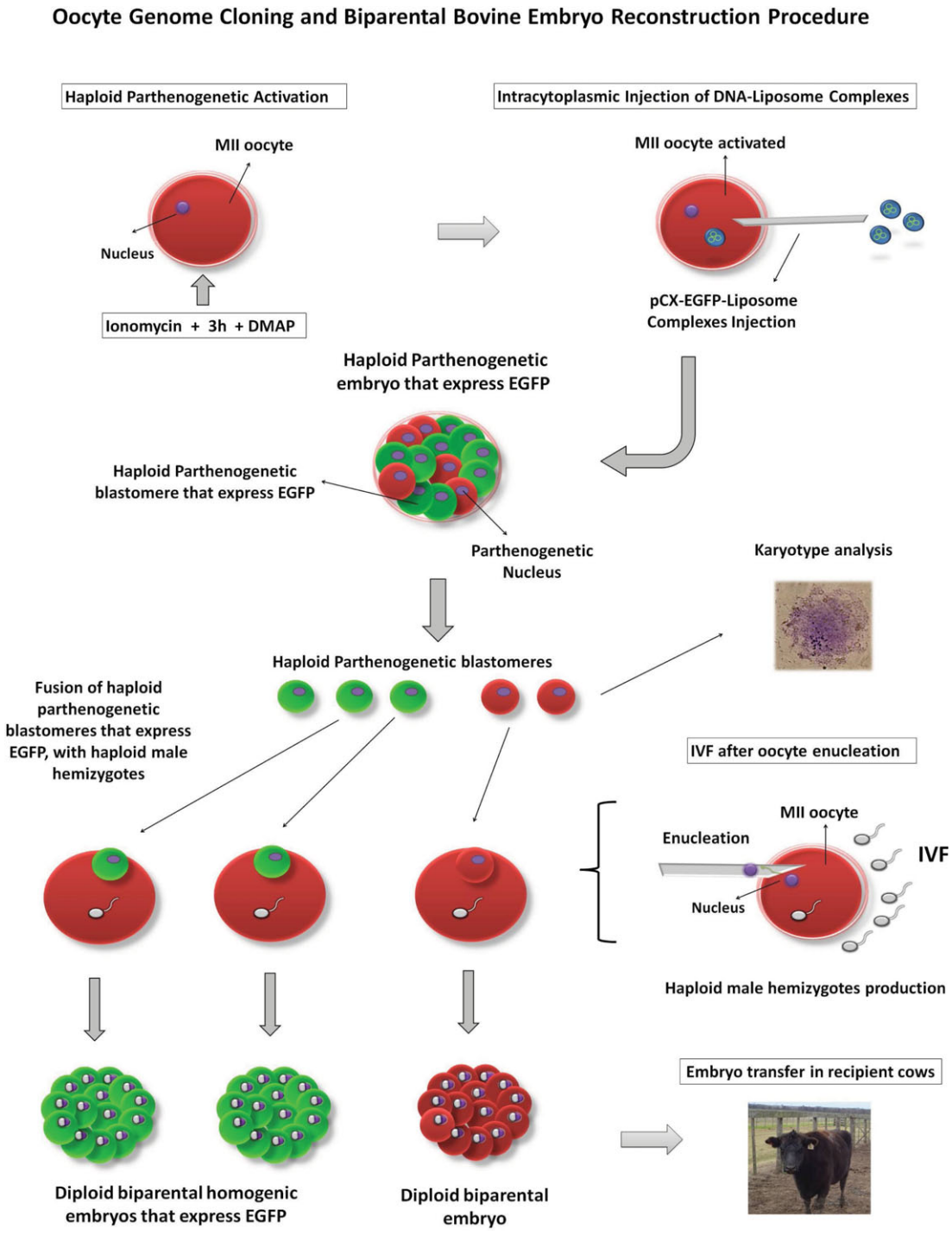

Figure 1 Schematic diagram showing the production of haploid parthenogenetic embryos and the biparental embryo reconstruction procedures. (See online for a colour version of this figure.)

mounted immediately between coverslips to count total nuclei under an epifluorescence microscope.

Immunocytochemical analysis

Immunocytochemical analysis was performed on bovine blastocysts, obtained as result of biparental embryo reconstruction ($n = 2$), and on IVF bovine blastocyst controls ($n = 3$). Briefly, embryos were fixed for 30 min in 4% v/v paraformaldehyde in PBS and permeabilized by 15 min incubation in PBS that contained 0.2% v/v Triton X-100. Non-specific immunoreactions were blocked by incubation with 3% v/v FBS and 0.1% v/v Tween-20 (Promega,

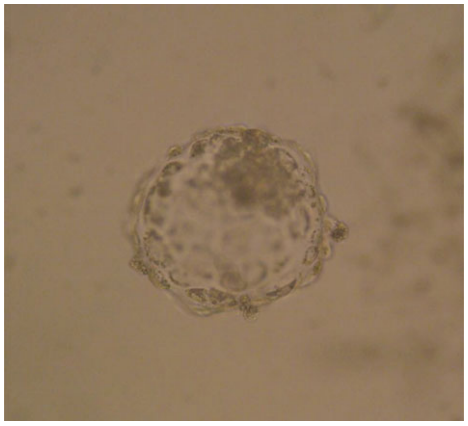

Figure 2 Biparental bovine blastocyst produced by fusion of a parthenogenetic haploid blastomere with a haploid male hemizygote and cultured in the well of the well system. Original magnification ×200. (See online for a colour version of this figure.)

H5152) in PBS (blocking buffer) for 30 min. After this pretreatment, affinity-purified primary polyclonal antibody against Oct-4 (Santa Cruz Biotechnology, California, USA) was diluted 1:100 in PBS and applied for 1 h at room temperature. Blastocysts were washed extensively in blocking buffer for 15 min. Then, the samples were incubated with secondary Alexa 488–donkey anti-goat IgG 2 mg/ml (Molecular Probes, Inc. Eugene, USA) diluted 1:1000 for 40 min at room temperature in the dark. After additional washing, the embryos were incubated in PBS that contained propidium iodide for 10 min in the dark. Embryos were mounted on slides in 70% v/v glycerol. Oct-4 negative controls were produced using only the secondary antibody. The embryos were analyzed on a Nikon confocal laser scanning microscope. An excitation wavelength of 488 nm was selected from an argon-ion laser to excite the Alexa-conjugated secondary antibody and a 544 nm wavelength to excite propidium iodide. Images of serial optical sections were recorded every 1.5 to 2 μm along the Z-axis of each embryo. Three-dimensional images were constructed using software EZ-C1 2.20.

Transfer of embryos

Reconstructed embryos were transferred to Aberdeen Angus recipients on day 7 of the oestrous cycle. Reconstructed embryos at the blastocyst stage (day 7) were used for embryo transfer. Each reconstructed embryo was washed several times in TL-HEPES and loaded into a 0.25 ml straw. Fresh embryos were transported to the farm at 35°C within 3 h. Embryos were transferred non-surgically to the uterine horn ipsilateral to the ovary bearing the corpus luteum, using a transverse hole-type transfer device. Each recipient received one embryo. Pregnancies were diagnosed by fetal membrane palpation through rectal inspection at approximately days 60–70 after oestrus.

Experimental design

In the first experiment we compared the developmental ability of parthenogenetic haploid and diploid bovine embryos produced with Io, Io + 3h + DAMP or Io + DMAP. For the best parthenogenetic haploid treatments (Io + 3h + DAMP) we injected a mixture of pCX–EGFP–liposome complexes 3 h following activation, in order to obtain exogenous gene expression to be used as a parthenogenetic cytoplasmic marker. Karyotype analysis was done in order to determine the ploidy of the parthenogenetic embryos and male hemizygotes generated. In the second experiment, haploid parthenogenetic embryos (4–16 cells) either expressing EGFP or not, were disaggregated and the parthenogenetic haploid blastomeres obtained were fused with zona-free haploid male hemizygotes in order to reconstruct biparental embryos. The developmental ability of the reconstructed embryos and the blastocyst cell numbers were evaluated. Additionally, the Oct-4 expression pattern of blastocysts obtained from biparental embryo reconstruction was analyzed by immunocytochemistry. Finally, reconstructed biparental embryos were transferred to recipient cows on day 7 of *in vitro* development. The procedure is shown in Fig. 1.

Statistical analysis

In vitro embryo development and transgene expression were compared by non-parametric Fisher's exact test. For all statistical analyses, the SAS program was used (SAS Institute, 1989). Differences were considered significant at $P < 0.05$.

Results

Experiment 1: Development of haploid parthenogenetic embryos, injected or not, with pCX–EGFP–liposome complexes

Development and EGFP-expression rates of haploid parthenogenetic embryos are summarized in Table 1. Statistical differences in cleavage rates were observed between the Io + 3h + DMAP group and the Io group, but not with the Io + 3h + DMAP injected with pCX–EGFP–liposome group. No differences were observed in rates of blastocyst development between the Io + 3h + DMAP and the Io + 3h + DMAP injected with pCX–EGFP–liposome groups, but significant differences were observed between the Io + 3h + DMAP

Table 1 Parthenogenetic haploid bovine embryo development and transgene expression

Parthenogenetic haploid activation treatment	Replicates	Injection of pCX–EGFP–liposome	*n*	Cleaved (%)	Morulae (%)	Blastocysts (%)	Blastocysts + EGFP embryos 4–16 cell (%)
Io	3	–	67	15 (22.4)[a]	1 (1.5)[a]	0 (0)[a]	NA
Io + 3h + DMAP	3	–	118	103 (87.3)[b]	23 (19.4)[b]	15 (12.8)[b]	NA
Io + 3h + DMAP	3	+	64	54 (84.4)[b]	33 (51.6)[c]	7 (10.9)[b]	35 (54.7)
Diploid activation control (Io + DMAP)	3	–	122	108 (88.5)[b]	53 (43.4)[c]	49 (40.2)[c]	NA

[a–d]Values with different superscripts in a column are significantly different ($P < 0.05$, Fisher's test). +, injected; –, not injected; DMAP, 6-dimethylaminopurine; EGFP, enhanced green fluorescent protein; Io, ionomycin; NA, not applicable.

Table 2 Development and transgene expression (+ EGFP) of bovine embryos reconstructed by parthenogenetic blastomere fusion

Method of production	Replicates	Parthenogenetic blastomere	*n*	Fused	Cleaved (%)	Blastocysts (%)	+ EGFP Embryos 2–16 cell (%)	+ EGFP in all embryo cells%
Biparental embryo	4	–EGFP	146	131 (89.7)	80 (61.1)	11 (8.4)[a]	NA	NA
Biparental embryo reconstructed	2	+EGFP	38	37 (97.4)	29 (78.4)	4 (10.8)[a,b]	29 (100)	28/29 (96.6)
Biparental IVF control	4	–EGFP	98	NA	63 (64.3)	22 (22.5)[b]	NA	NA

[a–d]Values with different superscripts in a column are significantly different ($P < 0.05$, Fisher's test). +, injected; –, not injected; DMAP, 6-dimethylaminopurine; EGFP, enhanced green fluorescent protein; Io, ionomycin; IVF, *in vitro* fertilization; NA, not applicable

and the Io group. All haploid parthenogenetic groups showed statistical differences in blastocyst rates compared with the diploid parthenogenetic control 40.2% (49/122), but no differences were seen in cleavage rates. The EGFP-expression pattern was evaluated in cleaved embryos (day 3 post activation). Parthenogenetic haploid embryos obtained by Io + 3h + DMAP treatment and then injected with pCX–EGFP–liposome complexes showed an EGFP-expression rate of 54.7% (35/54). Moreover, karyotype analysis of parthenogenetic embryos generated by Io + 3h + DMAP, confirmed that 83.3% (10/12) were indeed haploid. The remaining embryos were mixoploid. Karyotype analysis performed on male hemizygotes confirmed that 80.0% (8/10) of them were indeed haploid. The remaining male hemizygotes were diploid.

Experiment 2: Development and transgene expression of biparental bovine embryos reconstructed by fusion of parthenogenetic haploid blastomeres (EGFP-positive or -negative), with haploid male hemizygotes.

Development and EGFP-expression rates of ZP-free biparental embryos reconstructed with parthenogenetic haploid blastomeres that were either positive or negative for transgene expression, are summarized in Table 2. No differences were observed between these groups in the percentages of fusion and development, while biparental IVF controls showed a significantly higher blastocyst rate. All embryos that were reconstructed with EGFP-positive parthenogenetic haploid blastomeres expressed the transgene during development (100%, 29/29) and 96.6% (28/29) of them showed expression in all its blastomeres. Immunocytochemical analysis to determine the Oct-4 expression pattern was positive for the inner cell mass (ICM) and the trophoblast in the blastocysts analyzed ($n = 2$). A similar result was seen in IVF control embryos. To determine the number of blastocyst cells, biparental reconstructed blastocysts ($n = 6$) and IVF control blastocysts ($n = 8$) were stained on day 8, resulting in an average of 72.8 ± 7.0 and 97.0 ± 7.3 cells, respectively.

Experiment 3: Embryo transfer in recipient cows.

Reconstructed embryos ($n = 2$) were transferred at the blastocyst stage to Aberdeen Angus recipients. One pregnancy was diagnosed by fetal membrane palpation through rectal inspection at approximately 60 days after embryo transfer.

Discussion

Generation of parthenogenetic haploid embryos allows obtaining several blastomeres as identical copies of a single oocyte genome (Surani *et al.*, 1986; Escribá

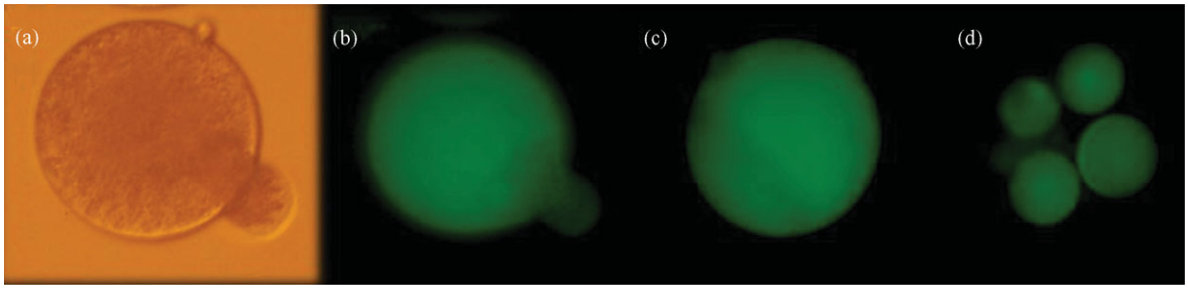

Figure 3 (*a*) ZP-free haploid male hemizygote fusing to an enhanced green fluorescent protein (+EGFP) parthenogenetic haploid blastomere (using phytohemagglutinin), prior to fusion. (*b*) The same zona pellucida (ZP)-free haploid male hemizygote fusing to a +EGFP parthenogenetic haploid blastomere under blue light. (*c*) The same ZP-free haploid male hemizygote completely fused to a +EGFP parthenogenetic haploid blastomere under blue light. (*d*) Biparental reconstructed ZP-free embryo expressing the transgene in all its blastomeres. The fluorescence was evaluated under blue light (488 nm). Original magnification ×200. (See online for a colour version of this figure.)

et al., 2001). Initially, we evaluated two methods to generate haploid parthenogenetic embryos, Io and Io + 3h + DMAP treatments. The parthenogenetic embryos produced by activation with only Io exposure, showed low cleavage rates and no blastocysts were obtained. However, parthenogenetic embryos produced by the Io + 3h + DMAP method cleaved and developed successfully regardless or not of whether they were injected with pCX–EGFP–liposome complexes (Table 1). Both these groups showed significant differences only in blastocyst development rates when compared with the diploid parthenogenetic control group. These results agree with previous reports that showed that haploid parthenogenetic embryos are compromised developmentally compared with diploid parthenogenetic embryos in different species such as cow, mouse and pig (Kaufman *et al.*, 1983; Henery *et al.*, 1992; Van De Velde *et al.*, 1999; Lagutina *et al.*, 2004). Recently, we demonstrated that intracytoplasmic injection of DNA–liposome complexes produces IVF and parthenogenetic embryos with an efficient expression of exogenous genes (Vichera *et al.*, 2010). In the present report, we also showed that haploid parthenogenetic embryos, produced by Io + 3h + DMAP and confirmed by karyotype analysis, cleaved successfully and showed high EGFP expression after injection of pCX–EGFP–liposome complexes (Table 1).

In the second experiment we demonstrated that it is possible to reconstruct biparental bovine embryos using female genome donors obtained from parthenogenetic haploid embryos up to the 16-cell stage (Table 2). Moreover, efficient fusion rates were obtained (90%), regardless of the embryonic stage of the donors (4–16 cells) and these embryos were capable of development to the blastocyst stage (Fig. 2). A previous report in mice observed that nuclear transfer of a maternal genome at the fourth cellular division, or more, in haploid male hemizygotes, severely impaired the developmental ability of the embryos generated (Surani *et al.*, 1986). This situation could be due to the existence of asynchrony between both parental genomes making a functional integration of both nuclei difficult.

All the biparental embryos reconstructed with EGFP-positive parthenogenetic haploid blastomeres expressed the transgene (100%) and most of them showed transgene expression in all blastomeres (96.6%). Development was not affected in this experiment, which suggested that transgene expression does not compromise *in vitro* embryonic progression. Expression of EGFP verified the cytoplasmic contribution of parthenogenetic haploid blastomeres in the reconstructed embryos (Fig. 3). On the other hand, positive expression of Oct-4 observed both in the ICM and the trophoblast in the blastocysts analyzed, as well as in the IVF control group embryos, was consistent with appropriate nuclear programming, agreeing with previous reports (Kirchhof *et al.*, 2000). In this study, statistical differences were found in cell numbers, between reconstructed biparental blastocysts and control IVF blastocysts. This finding indicates that the cell divisions kinetics could be modified by the biparental embryo reconstruction procedure.

Transfer of reconstructed embryos, at the blastocyst stage, to recipient cows, resulted in one pregnancy diagnosed by fetal membrane palpation through rectal inspection at approximately 60 days after embryo transfer. However this pregnancy was subsequently lost. Previous reports in mice showed that viable offspring could be produced by nuclear transfer of haploid parthenogenetic nuclei (Surani *et al.*, 1986). Offspring produced with this technique were not identical to one another nor to their parents, as variability is given by the parental counterpart. Moreover, live **o**ffspring were obtained from chimeras reconstructed from aggregation of parthenogenetic and *in vitro* fertilized bovine embryos (Boediono

et al., 1999). In the rabbit, oviductal transfer of reconstructed zygotes resulted in 100% pregnancy on day 12, but no pregnancies were diagnosed on day 21 after ovulation (Escribá *et al.*, 2001). One year later, the same authors obtained viable offspring when biparental embryos were reconstructed from cryopreserved haploid rabbit parthenotes (García-Ximénez *et al.*, 2002). These results indicate that the female gametic endowment can be successfully stored by cryopreservation of parthenogenetic haploid embryos. Germplasm cryopreservation makes the establishment of genetic banks possible for the conservation of biodiversity and contributes to the preservation of endangered species. In the future, the cryopreservation of parthenogenetic haploid embryos could be a very attractive option to maximize the conservation of genetic resources due to its greater resistance compared to oocyte cryopreservation.

In this work, we demonstrated that it is possible to multiply the haploid oocyte genome from a single bovine oocyte and that this haploid oocyte genome replicate can be used to generate biparental bovine embryos. Future research might consider the generation of stable haploid parthenogenetic cell lines as an alternative source of female gametes. The generation of parthenogenetic embryos has been achieved in several mammalian species (Kaufman *et al.*, 1983; Machaty *et al.*, 1997; Loi *et al.*, 1998; Liu *et al.*, 2002; Grabiec *et al.*, 2007; Méo *et al.*, 2007; Revazova *et al.*, 2007). Previously, karyotypically stable cell lines that maintain a haploid karyotype have been isolated from amphibians and insects (Freed *et al.*, 1970; Debec *et al.*, 1984). More recently the generation of haploid embryonic stem cells was described in Mekada fish (Yi *et al.*, 2009). This situation opens the possibility for oocyte genome cloning by multiplying the parthenogenetic haploid line. This would have the potential to generate an unlimited number of biparental embryos by combining these female haploid cells with haploid male hemizygotes to create a new combination of genetic traits from both parents.

In conclusion, we have shown that it is possible to obtain a consistent number of female haploid genome replicates from a single bovine oocyte and, subsequently, to use these replicates to generate biparental embryos. This approach offers enormous potential for livestock production as the use of genetic markers could allow the selection of certain favorable attributes prior to embryo reconstruction. Biparental embryo reconstruction by fusion of haploid oocyte genome replicates also could improve transgenic animal production because, in addition to increasing the number of transgenic embryos produced from a single oocyte, it also generates homogeneous transgene-expressing embryos. The capacity to alter the genome, by the introduction of exogenous genes with high efficiency and homogeneous expression, could increase the generation of transgenic farm animals that are useful in the pharmaceutical industry, in biomedicine or for livestock production.

Acknowledgements

The authors are grateful to CIALE for provision of the biological material. The authors thank Elizabeth Crichton and Lic Lucia Moro for their assistance with English. This work was supported by the Agencia de Promoción Científica y Tecnológica (PICT Redes N° 35142 2005–2009) and by Universidad de Buenos Aires (UBACYT G808).

References

Barton, S.C., Surani, M.A. & Norris, M.L. (1984). Role of paternal and maternal genomes in mouse development. *Nature* **311**, 374–6.

Bavister, B.D. & Yanagimachi, R. (1977). The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa *in vitro*. *Biol. Reprod*. **16**, 228–37.

Bhak, J.S., Lee, S.L., Ock, S.A., Mohana Kumar, M.B., Choe, S.Y. & Rho, G.J. (2006). Developmental rate and ploidy of embryos produced by nuclear transfer with different activation treatments in cattle. *Anim. Reprod. Sci.* **92**, 37–49.

Boediono, A., Suzuki, T., Li, L.Y. & Godke, R.A. (1999). Offspring born from chimeras reconstructed from parthenogenetic and in vitro fertilized bovine embryos. *Mol. Reprod. Dev.* **53**, 159–70.

Cibelli, J., Stice, S.L., Gluekey, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de León, A. & Robl, J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblast. *Science* **280**, 1256–8.

Collas, P., Fissore, R., Robl, J.M., Sullivan, E.J. & Barnes, F.L. (1993). Electrically induced calcium elevation, activation and parthenogenetic development of bovine oocytes. *Mol. Reprod. Dev.* **34**, 212–23.

Debec, A. (1984). Evolution of karyotype in haploid cell lines of *Drosophila melanogaster*. *Exp. Cell Res.* **151**, 236–46.

Escribá, M.J. & García-Ximénez, F. (1999). Electroactivation of rabbit oocytes in an hypotonic pulsing medium and parthenogenetic *in vitro* development without cytochalasin B-diploidizing pre-treatment. *Theriogenology* **51**, 963–73.

Escribá, M.J. & García-Ximénez, F. (2000). Influence of sequence duration and number of electrical pulses upon rabbit oocyte activation and parthenogenetic development. *Anim. Reprod. Sci.* **59**, 99–107.

Escribá, M.J. & García-Ximénez, F. (2001). Reconstruction of the heteroparental diploid condition in rabbit zygotes by nuclear transfer. *Theriogenology* **55**, 771–84.

Freed, J.J. & Mezger-Freed, L. (1970). Stable haploid cultured cell lines from frog embryos. *Proc. Natl. Acad. Sci. USA* **65**, 337–44.

García-Ximénez, F. & Escribá, M.J. (2002). Viable offspring derived from cryopreserved haploid rabbit parthenotes. *Theriogenology* **57**, 1319–25.

Grabiec, A., Max, A. & Tischner, M. (2007). Parthenogenetic activation of domestic cat oocytes using ethanol, calcium

ionophore, cycloheximide and a magnetic field. *Theriogenology* **67**, 795–800.

Hagemann, L.J. & First, N.L. (1992). Embryonic cytoplasmic extracts rescue murine androgenomes to the blastocyst stage. *Development* **114**, 997–1001.

Henery, C.C. & Kaufman, M.H. (1992). Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. *Mol. Reprod. Dev.* **31**, 258–63.

Ikawa, M., Kominami, K., Yoshimura, Y., Tanaka, K., Nishimune, Y. &, Okabe, M. (1995). A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). *FEBS Letts.* **375**, 125–8.

Kaufman, M.H. (1983). *Early Mammalian Development: Parthenogenetic Studies.* Cambridge, UK: Cambridge University Press pp. 84–164.

Kaufman, M.H. & Gardner, R.L. (1974). Diploid and haploid mouse parthenogenetic development following in vitro activation and embryo transfer. *J. Embryol. Exp. Morphol.* **31**, 635–42.

Kaufman, M.N., Lee, K.K.H. & Speirs, S. (1989). Post-implantation development and cytogenetic analysis of diandric heterozygous diploid mouse embryos. *Cytogenet. Cell Genet.* **52**, 15–8.

Kirchhof, N., Carnwath, J.W., Lemme, E., Anastassiadis, K., Schöler, H. & Niemann, H. (2000). Expression pattern of Oct-4 in preimplantation embryos of different species. *Biol. Reprod.* **63**, 1698–705.

Lagutina, I., Lazzari, G., Duchi, R. & Galli, C. (2004). Developmental potential of bovine androgenetic and parthenogenetic embryos: a comparative study. *Biol. Reprod.* **70**, 400–5.

Latham, K.E., & Solter, D. (1991). Effect of egg composition on the developmental 568 capacity of androgenetic mouse embryos. *Development* **113**, 561–8.

Liu, C.T., Chen, C.H., Cheng, S.P. & Ju, J.C. (2002). Parthenogenesis of rabbit oocytes activated by different stimuli. *Anim. Reprod. Sci.* **70**, 267–76.

Loi, P., Ledda, S., Fulka, J. Jr., Cappai, P., & Moor, R.M. (1998). Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. *Biol. Reprod.* **58**, 1177–87.

MacGrath, J. & Solter, D. (1983). Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. *Science* **220**, 1300–3.

MacGrath, J., & Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179–83.

Machaty, Z., Wang, W.H., Day, B.N. & Prather, R.S. (1997). Complete activation of porcine oocytes induced by the sulfhydryl reagent, thimerosal. *Biol. Reprod.* **57**, 1123–7.

Méo, S.C., Yamazaki, W., Ferreira, C.R., Perecin, F., Saraiva, N.Z., Leal, C.L. & Garcia, J.M. (2007). Parthenogenetic activation of bovine oocytes using single and combined strontium, ionomycin and 6-dimethylaminopurine treatments. *Zygote* **15**, 295–306.

Miki, H., Hirose, M., Ogonuki, N., Inoue, K., Kezuka, F., Honda, A., Mekada, K., Hanaki, K. I., Iwafune, H., Yoshiki, A., Ishino, F. & Ogura, A. (2009). Efficient production of androgenetic embryos by round spermatid injection. *Genesis* **47**, 155–60.

Obata, Y., Ono, Y., Akuzawa, H., Kwon, O.Y., Yoshizawa, M. & Kono, T. (2000). Post- implantation development of mouse androgenetic embryos produced by in-vitro fertilization of enucleated oocytes. *Hum. Reprod.* **15**, 874–80.

Revazova, E.S., Turovets, N.A., Kochetkova, O.D., Kindarova, L.B., Kuzmichev, L.N., Janus, J.D. & Pryzhkova, M.V. (2007). Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells* **9**, 432–49.

Rho, G.J., Wu, B., Kawarsky, S., Leibo, S.P. & Betteridge, K.J. (1998). Activation regimens to prepare bovine oocytes for intracytoplasmic sperm injection. *Mol. Reprod. Dev.* **50**, 485–92.

Salamone, D., Barañao, L., Santos, C., Bussmann, L., Artuso, J., Werning, C., Prync, A., Carbonetto, C., Dabsys, S., Munar, C. *et al.* (2006). High level expression of bioactive recombinant human growth hormone in the milk of a cloned transgenic cow. *J. Biotechnol.* **124**, 469–72.

SAS Institute Inc. *SAS/STAT User's Guide. Version 6. 4th Edn.* (1989). SAS Institute Inc. Cary NC, USA 943 pp.

Surani, M.A.H, Barton, S.C. & Norris, M.L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature Lond.* **308**, 548–50.

Surani, M.A.H., Barton, S.C. & Norris, M.L. (1986). Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell* **45**, 127–36.

Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Northey, D.L., Schutzkus, V. & First, N.L. (1994). Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. *Dev. Biol.* **166**, 729–39.

Vajta, G., Peura, T.T., Holm, P., Paldi, A., Greve, T., Trouson, A.O. & Callesen, H. (2000). New method for culture of zona-included or zona-free embryos: the well of the well (WOW) system. *Mol. Reprod. Dev.* **55**, 256–64.

Van De Velde, A., Liu, L., Bols, P.E., Ysebaert, M.T. & Yang, X. (1999). Cell allocation and chromosomal complement of parthenogenetic and IVF bovine embryos. *Mol. Reprod. Dev.* **54**, 57–62.

Vichera, G., Alfonso, J., Duque, C.C., Silvestre, M.A., Pereyra-Bonnet, F., Fernandez-Martín, R. & Salamone, D. (2009). Chemical activation with a combination of ionomycin and dehydroleucodine for production of parthenogenetic, ICSI and cloned bovine embryos. *Reprod. Dom. Anim.* **45**, e306–12.

Vichera, G., Moro, L. & Salamone, D. (2010). Efficient transgene expression in IVF and parthenogenetic bovine embryos by intracytoplasmic injection of DNA–liposome complexes. *Reprod. Domest. Anim.* **46**, 214–20.

Wells, D.N., Misica, P.M. & Tervit, H.R. (1999). Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* **60**, 996–1005.

Yi, Y.J. & Park, C.S. (2005). Parthenogenetic development of porcine oocytes treated by ethanol, cycloheximide, cytochalasin B and 6-dimethylaminopurine. *Anim. Reprod. Sci.* **86**, 297–304.

Yi, M., Hong, N. & Hong, Y. (2009). Generation of medaka fish haploid embryonic stem cells. *Science* **326**, 430–3.