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TESIS DOCTORAL

Effect of Origin and Culture Conditions on the Heterogeneity of Pluripotent Cell Populations

Efecto del origen y las condiciones de cultivo en la heterogeneidad de poblaciones celulares pluripotentes

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Priscila Ramos Ibeas

Directores

Alfonso Gutiérrez Adán
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Los doctores Alfonso Gutiérrez Adán y Miguel Ángel Ramírez de Paz, Investigadores Titulares del Departamento de Reproducción Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) hacen constar:

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Index / Índice

Abbreviations / Abreviaturas

2i	Two kinase inhibitors
ALH	Amplitude of lateral head displacement
ART	Assisted reproductive technologies
ASC	Adult stem cell
BBT	Bovine biopsied trophoblast
BEF	Bovine embryonic fibroblast
BES	Base excision repair
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMP	Bone morphogenetic protein
BNC	Binucleate cell
BOEC	Bovine oviductal epithelial cell
COC	Cumulus oocyte complex
Ct	Cycle threshold
DFS	DNA fragmented sperm
DMEM	Dulbecco´s modified Eagle medium
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase
Dpc	Days post coitum
EB	Embryoid body
ECC	Embryonal carcinoma cell
eCG	Equine chorionic gonadotropin
EDTA	Ethylenediaminetetraacetic acid
EF	Embryonic fibroblast
EGC	Embryonic germ cell
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EpiSC	Epiblast stem cell
ESC	Embryonic stem cell
ExE	Extraembryonic ectoderm
FCS	Foetal calf serum
FGF4	Fibroblast Growth Factor 4
GDNF	Glial cell-derived neurotrophic factor
GSC	Germline stem cell
GSK3	Glycogen synthase kinase 3
hAFSC	Human amniotic fluid stem cell
hCG	Human chorionic gonadotropin
HSC	Hematopoietic stem cell
IAP	Intracisternal-A particle
ICM	Inner cell mass

ICSI	Intracytoplasmic sperm injection
iPSC	Induced pluripotent stem cell
ISAS	Integrated semen analysis system
IVC	*In vitro* cultured
IVF	*In vitro* fertilized
KSR	Knockout serum replacement
LIF	Leukemia inhibitory factor
LTR	Long terminal repeat
MAPK/MEK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEG	Maternally expressed gene
mGSC	Multipotent germline stem cell
MNC	Mononucleate cell
MSC	Mesenchymal stem cell
NES	Nucleotide excision repair
PBS	Phosphate-buffered saline
PGC	Primordial germ cell
PI	Propidium iodide
PMSG	Pregnant mare serum gonadotropin
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SCF	Stem cell factor
SCNT	Somatic cell nuclear transfer
SNP	Single nucleotide polymorphism
SOF	Synthetic oviduct fluid
SPV	Smoothed path velocity
SSC	Spermatogonial stem cell
STR	Straightness ratio of VSL/VAP
TE	Trophectoderm
TET	Ten-eleven translocation
TSC	Trophoblast stem cell
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TV	Track velocity
Vs.	*Versus*
VSELs	Very small embryonic stem cells
XCI	X chromosome inactivation

Abstract

Pluripotent cells have fascinated the society since they were first discovered, and much research has been performed on them as they constitute a powerful tool for regenerative medicine or genetic manipulation. However, basic research is necessary to determine the optimal conditions for their identification, isolation and *in vitro* culture. Thus, in this thesis, several crucial aspects for cell lines derivation, such as the effect of embryonic source or culture conditions, or the origin of pluripotent cells, have been analyzed.

Embryonic stem cells (ESCs) have been widely used for research, and ESCs isolation techniques and culture systems have evolved in the last years improving derivation efficiency. Nevertheless, it remains controversial whether embryonic characteristics have an influence over the process. Some studies have described that modifications or alterations present in the original embryonic cells can be transmitted to their corresponding ESCs lines. However, in other circumstances, embryonic characteristics are not reflected in ESCs lines. In chapter I, the potential to derive ESCs lines from bad quality embryos was analyzed, and it was investigated whether these ESCs lines reflect some of the characteristics previously observed in such embryos.

Assisted reproductive technologies (ARTs) have been widely performed in humans and animals. However, embryo manipulation and *in vitro* culture is associated with perturbations of the embryonic ultrastructure and genetic and epigenetic alterations that may result in long-term effects, causing syndromes and diseases during adulthood. Especially worrying is the case of intracytoplasmic sperm injection (ICSI), as it bypasses the natural selection of spermatozoa during fertilization, allowing sperm with fragmented or damaged DNA to fertilize an oocyte. In this perspective, ICSI using DNA-fragmented sperm (DFS-ICSI) in mice was used as a model to generate bad quality embryos for ESCs lines derivation in chapter I of this thesis. DFS-ICSI embryos showed a reduced potential to generate ESCs lines compared to *in vivo*-produced embryos. Furthermore, during early passages, these DFS-ICSI-ESCs differed from *in vivo*-ESCs in the expression of certain genes related to pluripotency and epigenetic repression, DNA damage and repair, and *de novo* DNA methyltransferases and histone deacetylases. Gene deregulation in ESCs could reflect the alterations previously described in DFS-ICSI-generated embryos and adult animals However, at late passages, DFS-ICSI and *in vivo*-ESCs adopted similar expression profiles. Consequently, ESCs retain some memory of the embryos from which they were derived, although continuous passaging directs them to adopt similar profiles.

Furthermore, in chapter I it was observed that DFS-ICSI reduces sperm production and fertility in the male progeny, and affects the postnatal expression of a defined epigenetically sensitive allele, and that this modification may be inherited across generations.

Culture conditions constitute another main factor affecting cell lines derivation and features. In chapter II, the effect of culture conditions was analyzed during ESCs lines derivation. First, the effect of Leukemia inhibitory factor (LIF) supplementation during preimplantational embryo culture up to the blastocyst stage was analyzed. LIF, which is an essential factor for ESCs derivation and maintenance, could favor the transition from embryonic cells to ESCs. Although the embryos supplemented with LIF showed a lower total cell number, the ratio of inner cell mass (ICM)/total cells in blastocysts was significantly higher. Furthermore, these embryos proved to be more suitable for ESCs isolation, as their ESCs derivation efficiencies were higher. Additionally, blastocysts were cultured over a feeder layer of mouse embryonic fibroblasts (EF) in medium supplemented with: fetal calf serum (FCS) and LIF (ES medium); FCS, LIF and germline stem cells (GSCs)-related growth factors (GS medium); or FCS, LIF and MEK and GSK3β inhibitors (2i medium). We could observe an improvement in ESCs derivation efficiency when GS medium was used compared to ES medium. ESCs lines derived after LIF supplementation up to blastocyst stage and posterior culture in GS medium (LIF+GS) fulfilled pluripotency criteria and presented a higher expression of the imprinted gene *Meg3,* whose expression levels have been associated with chimeric animals formation ability in induced pluripotent stem cells (iPSCs). *Meg3* overexpression correlated to higher ESCs derivation efficiency and better chimeric animals formation ability. Thus, a possible synergy between LIF supplementation during embryo culture and the posterior addition of growth factors present in GS medium was observed that favours ESCs derivation. Therefore, ESCs derivation efficiency is highly dependent on culture medium. In the same way, transcriptome is defined by culture conditions, as gene expression in ESCs lines varied according to the different culture media employed for their derivation (ES, GS and 2i). Indeed, it has been reported that culture conditions are the major aspect determining gene expression, over embryonic origin and derivation procedure.

Historically, murine ESCs were equated to ICM cells because they were first produced from 3.5 dpc mouse blastocysts. However, several evidences based on the similarities existing among ESCs and embryonic germ cells (EGCs), the expression of key pluripotency genes in primordial germ cells (PGCs), or the fact that germ-cell markers are expressed in pluripotent cells indicate a different origin for ESCs. One of the most interesting theories states that a particular subpopulation of epiblast cells predisposed to differentiate towards the germ cell lineage is selected during the derivation process, giving rise to ESCs.

In chapter II, blastocysts were cultured in a germ cell-specific (GS) medium to favor ESCs lines derivation through a possible germ cell-like intermediate state. ESCs derivation efficiency was significantly higher in GS medium than in regular ES medium. The expression of germ cell specific genes was detected in all conditions, although there was

no evidence for a shift towards germ cell specification induced by GS medium, as we found germ cell-specific genes expression in all culture conditions (GS, ES and 2i). The appearance of this common germ cell-like intermediate state has been reported in other articles that used standard FCS culture conditions for ESCs derivation, but it seems to be facultative for the stabilization of pluripotency *in vitro,* since culture in 2i conditions without FCS or EFs enables the effective direct recruitment of ESCs skipping this step. Consequently, it could be possible that the intermediate germ cell-like state is induced by FCS or by other factors secreted by murine EFs.

Multipotent stem cells could also present this germ cell-like state, as it has been demonstrated that RNA processing pattern in certain stem cells is similar to the testicle, and the expression of germline-specific genes has been detected in adult tissues containing multipotent cells populations. In chapter III, we studied the expression of the germline-specific gene *Dazl* throughout development by using a *Dazl*-eGFP-transgenic mouse. Preimplantational embryos, foetal, neonatal and adult tissues were analyzed for *Dazl*-driven-eGFP expression that could indicate the presence of pluripotent cells. During preimplantational embryo development, *Dazl*-eGFP was detected from zygote to blastocysts. Although *Dazl*-eGFP expression was localized mainly in the gonads during fetal development and in adulthood, it was also detected in other tissues as intestine and bone marrow. Interestingly, different multipotent cells populations reside in these tissues, such as intestinal stem cells and bone marrow mesenchymal stem cells. Supporting our results, other studies have reported the expression of germline-specific genes in mouse and human bone marrow. Thus, multipotent cells could share a common germ cell-like origin with other *in vitro* cultured pluripotent populations, and *Dazl*-eGFP transgene could be used to explore the presence of multipotent cells in different tissues.

In chapter IV, a system to derive bovine biopsy-derived trophoblastic cell lines was established. Two critical aspects for bovine trophoblastic cell lines establishment are embryo or biopsy adhesion velocity to the culture plate and a suitable culture medium. To date, most of the bovine trophoblastic cell lines have been derived by co-culture with mouse embryonic fibroblasts. We have developed a microdrop culture system over a gelatinized surface to enhance fast adhesion, and we have analyzed culture media conditioned by different cell lines as an alternative to co-culture, avoiding the risk of contamination with other cell types. Conditioned media from mouse embryonic fibroblasts (Cm), bovine embryonic fibroblasts (Cb) and bovine oviductal cells (Co) were assayed. Except for Cb, conditioned media improved derivation efficiency, being Cm the most efficient medium for trophoblastic cell lines derivation. High variability in gene expression patterns was observed in trophoblastic cell lines derived in the same conditions. These different gene expression patterns should be due to the embryonic source, confirming that trophoblastic cell lines derivation, like ESCs derivation, is affected

by embryonic characteristics. In the same way, transcriptome seems to be affected by long term culture as gene expression patterns varied along time in culture, indicating that trophoblastic cell lines are dynamic populations. Trophoblastic cell lines mimicked *in vivo* trophectoderm behaviour and showed characteristics previously described by other authors as mononucleate and binucleate cells presence and trophoblastic-specific genes expression. Furthermore, cell lines were able to proliferate for more than two years, and pluripotency-related genes expression was detected, revealing certain self-renewal capacity and the presence of a population of multipotent cells.

Resumen

Desde su descubrimiento, las células pluripotentes han fascinado a la sociedad y han sido ampliamente utilizadas en investigación debido a que constituyen una poderosa herramienta para la medicina regenerativa y para la manipulación genética. Existen todavía muchas lagunas sobre las condiciones óptimas para su identificación, aislamiento y cultivo *in vitro*. Por ello, en esta tesis se han analizado varios aspectos determinantes para la obtención de líneas celulares, como el efecto de la fuente embrionaria, las condiciones de cultivo o el origen de las células pluripotentes.

Las células troncales embrionarias ("embryonic stem cells", ESCs) son uno de los tipos de células pluripotentes más empleado, y las técnicas para su aislamiento y cultivo han evolucionado en los últimos años para mejorar su eficiencia de obtención, pero aún se desconoce si las características embrionarias influyen en su aislamiento. Estudios previos han descrito que las modificaciones o alteraciones presentes en las células embrionarias originales pueden ser transmitidas, en algunos casos, a sus correspondientes líneas de ESCs; sin embargo, en otras circunstancias no se ven reflejadas en las ESCs. En el capítulo I de esta tesis se ha analizado el potencial de embriones de "mala calidad" para dar lugar a líneas de ESCs, y si estas líneas de ESCs reflejan algunas de las características previamente observadas en dichos embriones.

Las técnicas de reproducción asistida ("assisted reproductive technologies", ARTs) han sido extensamente utilizadas en humanos y en animales. Sin embargo, la manipulación y el cultivo *in vitro* de los embriones se ha asociado con la aparición de alteraciones genéticas, epigenéticas y en la ultraestructura de los embriones que pueden dar lugar a síndromes y enfermedades durante la edad adulta. El caso de la técnica de inyección intracitoplasmática de espermatozoides ("intracytoplasmic sperm injection", ICSI) es en algunos casos especialmente preocupante, ya que franquea las barreras de selección natural del esperma, permitiendo que espermatozoides con ADN dañado o fragmentado fertilicen el ovocito. Por lo tanto, la técnica de ICSI utilizando espermatozoides con ADN fragmentado ("DNA-fragmented sperm", DFS-ICSI) en el ratón se ha utilizado como un modelo para generar embriones de "mala calidad" para la obtención de líneas de ESCs en el capítulo I de esta tesis. Los embriones generados por DFS-ICSI mostraron un menor potencial para generar líneas de ESCs que los embriones producidos *in vivo*. Además, en pases tempranos, estas líneas obtenidas mediante DFS-ICSI mostraron diferencias en la expresión de ciertos genes relacionados con la pluripotencia y la represión epigenética, el daño y la reparación del ADN, ADN metil-transferasas *de novo* y deacetilasas de histonas, en comparación con las ESCs procedentes de embriones producidos *in vivo*. Sin embargo, en pases tardíos las ESCs *in vivo* y las procedentes de DFS-ICSI adoptaron perfiles de expresión génica similares. Consecuentemente, las ESCs retienen cierta memoria de los embriones de los que proceden, aunque el cultivo a largo plazo hace que adopten perfiles similares.

Además, en el capítulo I se observaron otras alteraciones en los animales obtenidos mediante DFS-ICSI, como una reducción en la producción de esperma y en la fertilidad de los machos de la descendencia, y ciertas alteraciones en la expresión postnatal de un alelo sensible definido epigenéticamente, siendo esta modificación heredada transgeneracionalmente.

Las condiciones de cultivo son otro factor crítico que afecta a la obtención de líneas celulares y a sus posteriores características. En el capítulo II se ha analizado el efecto de las condiciones de cultivo durante la obtención de líneas de ESCs. En primer lugar se analizó el efecto de suplementar el medio con el factor inhibidor de leucemia ("Leukemia inhibitory factor", LIF) durante el cultivo del embrión preimplantacional hasta el estadio de blastocisto. LIF, que es esencial para las ESCs de ratón, podría favorecer la transición de las células embrionarias a ESCs. Aunque los embriones suplementados con LIF mostraron un menor número total de células, la proporción entre el número de células de la masa celular interna (ICM) y las células totales del blastocisto fue significativamente mayor. Además, estos embriones fueron más aptos para el aislamiento de ESCs, ya que mostraron una mayor eficiencia de obtención. Posteriormente, los blastocistos fueron cultivados sobre una monocapa de fibroblastos embrionarios ("embryonic fibroblasts", EF) murinos en medio suplementado con: suero fetal bovino ("fetal calf serum", FCS) y LIF (medio ES); FCS, LIF y factores de crecimiento utilizados para el cultivo de células troncales de linaje germinal ("germline stem cells", GSCs) (medio GS); o FCS, LIF e inhibidores de MEK y GSK3β (medio 2i). Pudimos observar un incremento en la eficiencia de obtención de ESCs en el medio GS en comparación con el medio ES. Las líneas obtenidas tras la adición de LIF durante el cultivo hasta blastocisto y el posterior empleo de medio GS (LIF+GS) presentaron un buen patrón de pluripotencia y una mayor expresión del gen de imprinting *Meg3*, cuyos niveles de expresión se han asociado con la capacidad de formación de animales quiméricos de las células de pluripotencia inducida ("induced pluripotent stem cells", iPSCs). Dicha sobre-expresión de *Meg3* correlacionó con una mayor eficiencia de obtención de ESCs y con una mayor capacidad de formación de ratones quiméricos. Se observó por lo tanto una posible sinergia entre la suplementación con LIF durante el cultivo embrionario *in vitro* y la adición posterior de otros factores de crecimiento presentes en el medio GS que favorece la creación de nuevas líneas de ESCs. Por ello, la eficiencia de obtención de ESCs depende en gran medida del medio de cultivo. Del mismo modo, el transcriptoma está determinado por las condiciones de cultivo, ya que la expresión génica de las líneas de ESCs varió de acuerdo a los diferentes medios de cultivo empleados para su obtención (ES, GS y 2i). De hecho, se ha descrito que las condiciones de cultivo son el factor más condicionante para la expresión génica de las células pluripotentes, por encima del origen embrionario o el procedimiento de obtención.

Históricamente las ESCs se han considerado equivalentes a las células de la ICM porque se obtuvieron por vez primera a partir de blastocistos murinos de día 3,5. Sin embargo, varias evidencias como la similitud existente entre las ESCs y las células germinales embrionarias ("embryonic germ cells", EGCs), la expresión de genes de pluripotencia en células primordiales germinales ("primordial germ cells", PGCs), o el hecho de que en las células pluripotentes se expresen marcadores de células germinales, indican que las ESCs podrían tener un origen diferente. Una de las teorías más interesantes acerca de ello indica que una subpoblación de células del epiblasto en concreto, predispuesta a diferenciarse hacia el linaje germinal, sería seleccionada durante el proceso para dar lugar a las ESCs.

En el capítulo II se cultivaron blastocistos en un medio específico de células germinales (medio GS) para favorecer la obtención de líneas de ESCs a través de este estado intermedio predispuesto a diferenciarse hacia el linaje germinal, y la eficiencia de obtención fue significativamente mayor en medio GS que en el medio ES tradicional. Sin embargo, no hubo resultados que indicaran una diferenciación más pronunciada hacia el linaje germinal en estas células inducida por el medio GS en concreto, ya que pudimos encontrar expresión de genes específicos del linaje germinal en todas las condiciones de cultivo (ES, GS y 2i), indicando la aparición de este estado similar a una célula germinal durante el proceso de obtención de ESCs en todas las condiciones analizadas. La aparición de este estado se ha descrito en otros artículos en los que se utilizaron condiciones de cultivo estándares con FCS para la obtención de ESCs; aunque parece no ser indispensable para la estabilización de la pluripotencia *in vitro*, ya que el cultivo en condiciones 2i (con los inhibidores de MEK y GSK3β) sin el uso de FCS o EFs, permite el reclutamiento directo y efectivo de ESCs saltándose este estado. Consecuentemente, podría ser posible que el estado intermediario similar a una célula germinal esté inducido por el FCS o por otros factores secretados por los EFs.

Por otra parte, las células multipotentes podrían mostrar también este estado similar a una célula germinal, ya que se ha demostrado que el patrón de procesamiento de RNA de ciertas células multipotentes es similar al del testículo; además, se ha encontrado expresión de genes específicos del linaje germinal en algunos tejidos adultos que contienen poblaciones de células multipotentes. En el capítulo III hemos estudiado el gen específico del linaje germinal *Dazl* mediante un ratón transgénico *Dazl*-eGFP, analizando el desarrollo embrionario preimplantacional y los tejidos fetales, neonatales y adultos en busca de expresión de eGFP inducida por *Dazl* que pudiera indicar la presencia de células pluripotentes. Durante el desarrollo embrionario preimplantacional, *Dazl*-eGFP fue detectado desde el estadio de cigoto hasta el de blastocisto. Aunque la expresión de *Dazl*-eGFP se localizó principalmente en las gónadas durante el desarrollo fetal y en la edad adulta, también se detectó en otros tejidos como el intestino y la médula ósea.

Curiosamente, en estos tejidos existen diferentes poblaciones de células multipotentes como las células troncales intestinales y las células troncales mesenquimales de la médula ósea. Otros estudios han descrito la expresión de genes específicos del linaje germinal en la médula ósea en humanos y en el ratón. Por ello, las células multipotentes podrían compartir un origen común similar a una célula germinal con otras poblaciones de células pluripotentes cultivadas *in vitro*, y el transgen *Dazl*-eGFP podría ser usado para explorar la presencia de células multipotentes en diferentes tejidos.

En el capítulo IV de esta tesis se ha establecido un sistema para obtener líneas celulares trofoblásticas a partir de biopsias embrionarias bovinas. Dos aspectos críticos para el establecimiento de líneas celulares trofoblásticas bovinas son la velocidad de adhesión del embrión o de la biopsia a la placa de cultivo, y el empleo de un medio de cultivo adecuado. Hasta la fecha, la mayoría de las líneas de células trofoblásticas bovinas han sido obtenidas mediante co-cultivo sobre una monocapa de fibroblastos embrionarios murinos. Nosotros hemos desarrollado un sistema de cultivo en microgota sobre una superficie gelatinizada para facilitar una rápida adhesión, y hemos analizado medios condicionados por diferentes líneas celulares como alternativa al cocultivo, evitando así el riesgo de contaminación con otros tipos celulares. Se ha analizado la eficiencia de obtención de líneas celulares en medios de cultivo condicionados por fibroblastos embrionarios murinos (Cm), fibroblastos embrionarios bovinos (Cb) y células oviductales ovinas (Co). A excepción del medio Cb, los medios condicionados mejoraron la eficiencia de obtención, siendo Cm el medio más eficiente para el aislamiento de líneas de células trofoblásticas. Se observó una alta variabilidad en los patrones de expresión génica entre las líneas de células trofoblásticas obtenidas en las mismas condiciones. Dichas diferencias en la expresión génica podrían estar debidos a la fuente embrionaria, confirmando que la obtención de líneas de células trofoblásticas, al igual que la obtención de ESCs, se encuentra afectada por las características embrionarias. Del mismo modo, el cultivo a largo plazo parece afectar al transcriptoma de estas líneas celulares, ya que los patrones de expresión génica variaron a lo largo de diferentes pases, lo que también indica que las líneas de células trofoblásticas son poblaciones dinámicas. Las líneas de células trofoblásticas generadas mostraron un comportamiento similar al del trofectodermo *in vivo* y características similares a las descritas previamente por otros autores como la presencia de células mononucleadas y binucleadas, y la expresión de genes específicos del trofoblasto. Además, las líneas celulares fueron capaces de proliferar durante más de dos años y se detectó expresión de genes relacionados con la pluripotencia, revelando cierta capacidad de auto-renovación o la presencia de una población de células multipotentes.

Introduction

Discovering pluripotency, a brief historical perspective

1. From the discovery of pluripotent cells to pluripotency capture *in vitro*.

Pluripotency and stem cells have fascinated both biologists and clinicians for over a century. The term stem cell was first employed in the scientific literature as early as 1868 by the eminent german biologist Ernst Haeckel, who used the term ‘‘Stammzelle’’ to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved (Haeckel 1868). Later, he proposed that the fertilized egg should also be called stem cell (Haeckel 1877). Thus, according to Haeckel, the term stem cell was used in two senses: as the unicellular ancestor of all multicellular organisms and as the fertilized egg that gives rise to all cells of the organism (Ramalho-Santos and Willenbring 2007).

In 1892, another german scientist, Theodor Boveri, took Haeckel’s definition of stem cell as the fertilized egg one step further: he proposed the term stem cell for the earliest germline originated in animal embryos, which would presumably carry the germ-plasm and would differentiate later into germ cells (Boveri 1892). Therefore, in these early studies, the term stem cell referred to what we today call germline, or primordial germ cells.

Four years later, the term was popularized in the English language by Edmund B. Wilson, an American scientist who reviewed Boveri’s studies in his famous book *The Cell in Development and Inheritance* (Wilson 1896). This book was inspirational to embryologists and geneticists of the time, and Wilson was generally credited as having coined the term stem cell.

Around the same time, research on the development and regeneration of the hematopoietic system was going on, and a group of scientists believed that a cell existed that represented the common origin of the various cell types of the blood. Some of them began to use the term stem cell to refer this common precursor (Pappenheim 1896, Ramalho-Santos and Willenbring 2007).

Thus, the first interpretations of stem cells in the late 19th century concerned fundamental questions in embryology: the continuity of the germline and the origin of the blood system.

The existence of hematopoietic stem cells was demonstrated afterwards (Till and Mc 1961, Becker, Mc et al. 1963, Till, McCulloch et al. 1964) and these cells were established

as the prototypical stem cells, capable of proliferating indefinitely (self-renewal) and of giving rise to specialized cells (differentiation) (Figure 1).

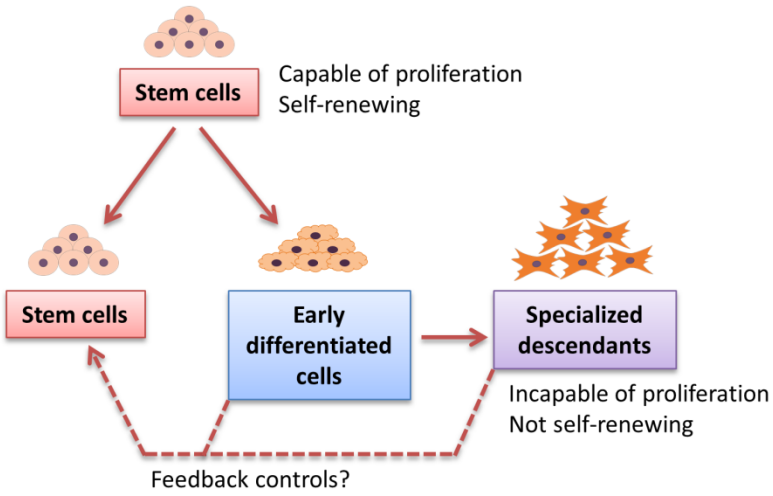

Figure 1. Representation of the modes of proliferation of blood-forming cells Adapted from (Till, McCulloch et al. 1964).

Bases for pluripotent cells culture were established through research in mouse teratocarcinomas. The discovery that male mice of strain 129 had a high incidence of testicular teratocarcinomas, and that their growth was sustained by embryonal carcinoma cells (ECCs) (Stevens and Little 1954) marked the beginning of experimentation with tumors by serially transplantation between mice. Subsequently, conditions for ECCs culture were developed, and they were established as an *in vitro* model for mammalian development (Kahan and Ephrussi 1970). Moreover, it was shown that ectopic transplantation of germinal ridges and of preimplantation embryos similarly induced teratocarcinoma formation; that is, by the transplantation of sources of pluripotent cells (Stevens 1967).

The next step was to attempt the direct derivation of pluripotent cell lines *in vitro,* and it was achieved from delayed blastocysts using the culture conditions previously established to support ECCs (Evans and Kaufman 1981). These cells were the first embryonic stem cells (ESCs) ever to be isolated. ESCs, unlike ECCs, proved to chimerize not only the soma but also the germline of the resulting mice, being efficient tools for genetic manipulation from culture to creature (Bradley, Evans et al. 1984, Robertson, Bradley et al. 1986) (reviewed in (Solter 2006)).

In 1992, pluripotent cell lines were isolated *in vitro* from a different source, primordial germ cells (PGCs), by growth factors supplementation to the regular ESCs culture medium (Matsui, Zsebo et al. 1992). These cells shared important characteristics with ESCs,

including morphology, pluripotency and germline transmission in chimera formation. They were called embryonic germ cells (EGCs), to distinguish them from ESCs derived from blastocysts.

Since murine ESCs were isolated for the first time, it took seventeen years until the isolation of human ESCs was announced (Thomson, Itskovitz-Eldor et al. 1998). This was probably due to the developmental differences existing between these species. However, pluripotency of these cells was lower than their murine counterparts, as they were not competent to contribute to blastocyst chimeras under standard culture conditions (it has been recently discovered that culture medium supplementation with certain factors increases human ESCs pluripotency, making them equivalent to murine ESCs and able to contribute to chimeras (Gafni, Weinberger et al. 2013)). Moreover, human ESCs formed flat colonies, resembling cell lines derived from mouse late epiblasts, termed epiblast stem cells (EpiSCs) (Tesar, Chenoweth et al. 2007), in contrast to dome-shaped colonies formed by mouse ESCs. Therefore, two states of pluripotency were proposed: **naïve pluripotency**, comprising rodent ESCs depending on LIF/Stat3 signaling, and **primed pluripotency**, comprising primate ESCs and rodent EpiSCs depending on Fgf/ERK signaling (Nichols and Smith 2009).

Although human ESCs derivation was recognized as a great medical advance, several ethical concerns raised about the employment of human embryos for research. These concerns were overcame by the discovery of the reprogramming factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) by Yamanaka *et al*., which allowed the reprogramming of somatic cells into pluripotent cells, generating mouse induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). Subsequently, human iPSCs were obtained omitting *c-Myc* and using *LIN28* as the fourth factor (Takahashi, Tanabe et al. 2007).

In summary, in the last decades different types of pluripotent cells obtained at diverse developmental stages have been proved to be cultured *in vitro* while maintaining self-renewal and pluripotency (Figure 2).

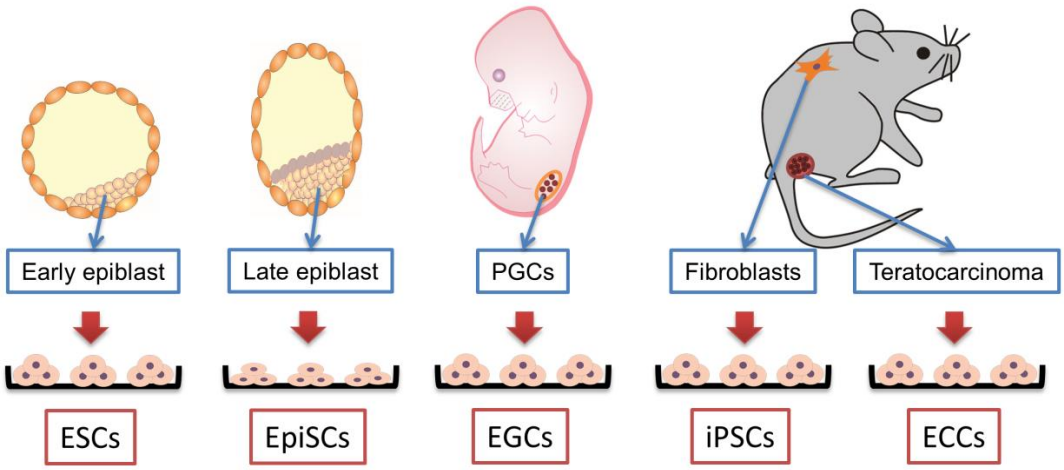

Figure 2. Pluripotent cell types. During preimplantation development, ESCs can be derived from early epiblasts from delayed blastocysts. Later, EpiSCs can be derived from late epiblasts from implanted embryos. Afterwards, EGCs can be isolated from PGCs in foetus, and during adulthood iPSCs can be obtained through somatic cell reprogramming and ECCs can be isolated from teratocarcinomas.

2. Different theories for the origin of embryonic stem cells

During development, a totipotent zygote gives rise to billions, even trillions, of differentiated cells in a coordinated manner to form an organism (Niwa and Fujimori 2010).

In vertebrates, the evolution of the embryo gives rise to both extraembryonic lineages and proembryonary cells. Proembryonary cells are pluripotent cells that maintain an undifferentiated state and show a high mitotic rate (Johnson 2001), and these properties lead directly to their ability to derive ESCs. According to the taxonomy and to the developmental stage, proembryonary cells can have different names. They are called inner cell mass in mice and later epiblast (Rossant 2001). In birds they are known as germ, then blastoderm and later epiblast (Eyal-Giladi and Kochav 1976), and their equivalent in marsupials is pluriblast and later epiblast (Johnson and Selwood 1996).

Morphology and distribution of ICM and TE depend on taxonomy. In marsupials both ICM and TE are superficial, establishing an unilaminar blastocyst. After hypoblast differentiation the blastocyst becomes bilaminar, being the epiblast and the TE superficial. In birds, the X stage of Hamburger and Hamilton is composed of two differentiated regions: the *area pellucida* that will form the embryo and a surrounding dark area (*area opaca*) that will form the extraembryonic yolk sac (Eyal-Giladi and Kochav 1976). The formation of the hypoblast starts in the X stage as well, and in the XI stage, the

area pellucida is composed of both the epiblast and the hypoblast. Thus, the X stage is used in birds for ESCs isolation and for gene targeting to produce chimeric animals. In zebrafish, medakafish and goldfish, after the tenth division (mid-blast), the zygotic genome is activated giving rise to the first three cell lineages. Two of them are extraembryonic lineages: the yolk sincitial layer, and the surrounding external layer, and the third layer is the pluriblast (inner cell layer) that will form the embryo (Fan, Crodian et al. 2004). In these three fish species, the mid-blast stage has been used for ESCs isolation, and the germinal ridge in zebrafish as well.

In mice, at 3.5 dpc (days post coitum), the blastocyst is composed of two lineages, the ICM and the trophectoderm (TE). The ICM gives rise to the primitive ectoderm or epiblast and to the primitive endoderm or hypoblast, while the TE contributes just to the placental tissues (Rossant 2001). After the differentiation of the hypoblast, the residual ICM turns into the early postimplantation epiblast, and these pluripotent cells quickly differentiate into the primary germ layers during the gastrulation. Historically, ESCs have been equated to ICM cells because they were first produced from 3.5 dpc mouse blastocysts. However, growing evidence indicates a different origin for mouse ESCs. Some years ago, a theory arose that a particular subpopulation of epiblast cells, selected during the derivation process, gives rise to ESCs, and an attractive candidate for this subpopulation were epiblast cells predisposed to differentiate towards the germ cell lineage (Zwaka and Thomson 2005). Supporting this thought, on one hand primordial germ cells (PGCs) can be induced to generate pluripotent cell lines (EGCs), which are indistinguishable from ESCs (Matsui, Zsebo et al. 1992, Resnick, Bixler et al. 1992), and on the other hand, among all lineages that develop from the epiblast, only germ cells recover the expression of pluripotency-related genes during their specification, such as *Oct4*, *Nanog* and *Sox2* (Durcova-Hills and Surani 2008, Chu, Surani et al. 2011). Furthermore, germ-cell specification factor *Blimp1* and other germ-cell markers, such as *Dppa3 (Stella)* and *Prdm14*, were shown to be activated in blastocysts explants cultured in regular ESCs conditions (medium supplemented with fetal calf serum and LIF) during ESC derivation (Chu, Surani et al. 2011), suggesting that cells committed to become ESCs transiently activate a transcriptional program specific for PGCs (Hochedlinger 2011). In fact, when cells upregulating *Blimp1* were sorted from ICM outgrowths and transplanted into 8.5 dpc germ cell-deficient embryos, they migrated to the genital ridges and upregulated the germline maturation marker *Mvh*. Moreover, these sorted cells gave rise to ESCs lines nine times more efficiently than bulk ICM cells did. Nevertheless, a germ-cell biased reprogramming does not seem to be strictly necessary for ESCs derivation, as ESCs lines could be derived from blastocysts deficient for *Blimp1* (Chu, Surani et al. 2011). Furthermore, ESCs derivation in 2i culture system (further explained afterwards in the introduction) does not result in *Blimp1* upregulation; thus, it does not involve a transitory

germ cell program, but directly captures epiblast cells self-renewal potential (Hochedlinger 2011).

Pluripotency tests and hallmarks

Pluripotent cells are defined by two characteristics: the capacity to divide indefinitely while maintaining the undifferentiated state or self-renewal, and the ability to differentiate towards any of the three germ layers (endoderm, mesoderm and ectoderm). Several *in vitro* and *in vivo* techniques are regularly used to validate pluripotency.

In vitro, naïve pluripotent cells grow as round dome-shaped colonies, while flat colonies are characteristic of primed pluripotent cells or of differentiation (Nichols, Silva et al. 2009, Nichols and Smith 2009).

One of the most reliable protocols used to detect pluripotency is alkaline phosphatase staining, as undifferentiated pluripotent cells show elevated levels of this enzime. Furthermore, a panel of biochemical and molecular markers has been identified that are specific to pluripotent cells and fundamental for maintaining the undifferentiated state. In mice, the main pluripotent markers are *Oct4*, *Nanog*, *Sox2* and *Fut4 (Ssea1)* (Marti, Mulero et al. 2013).

Furthermore, *in vivo* and *in vitro* differentiation tests can be performed. When allowed to differentiate *in vitro* through embryoid bodies (EBs), pluripotent cells form round compact cellular aggregates that grow in suspension and generate the three primitive embryonic layers (ectoderm, mesoderm and endoderm). In the same way, when injected into ectopic sites in host animals, pluripotent cells produce teratomas, which contain multiple types of differentiated tissue, representative of the three primitive embryonic layers *in vivo*. It is remarkable that not only fully pluripotent cells differentiate into the three embryonic layers, as human ESCs and murine EpiSCs generate teratomas and EBs as well (Garcia-Lavandeira et al. 2012).

The golden pluripotency hallmark lies in the generation of germline-competent chimaeras by combining host embryos with pluripotent cells. Chimaeras can be generated by the tetraploid complementation assay or by pluripotent cells microinjection. Tetraploid embryos are produced by fusing the two cells of an embryo at the two-cell stage by an electrical current. These embryos can develop normally to the blastocyst stage and tetraploid cells can form the extra-embryonic tissue; however, a proper fetus rarely develops. In the tetraploid complementation assay, a tetraploid embryo, either at the morula or blastocyst stage, is combined with diploid pluripotent cells, and the embryo then develops normally, being the fetus exclusively derived from the pluripotent cells,

while the extra-embryonic tissues are established by the tetraploid cells. The ability of contributing to the generation of chimaeras can be also tested after pluripotent cells microinjection into a host embryo in morulla or blastocyst stage (Ramirez, Fernandez-Gonzalez et al. 2009) or after pluripotent cells aggregation with an 8-cell embryo (Ramirez, Pericuesta et al. 2007). Only truly pluripotent cells are able to integrate into the embryo and to contribute to the formation of all organs of the animal and, more important, to the germline in order to be transmitted to following generations, and this property is characteristic of naïve pluripotent cells. On the contrary, primed pluripotent cells as mouse EpiSCs and ESCs in species as pig, bovine and human, are unable to contribute to chimeric animals, demonstrating that they are not fully pluripotent (Brevini, Antonini et al. 2008, Alvarez, Garcia-Lavandeira et al. 2012). However, some authors have described the generation of chimeric animals from naïve pluripotent cells in pig (Fujishiro, Nakano et al. 2013), human (Gafni, Weinberger et al. 2013) and sheep (Sartori, DiDomenico et al. 2012).

Influence of cellular or embryonic source on epigenetics and pluripotency

1. Epigenetic modifications

Epigenetic modifications comprise all changes in the chromatin that modify gene expression without altering the nucleotide sequence. The main epigenetic modifications are DNA methylation, which involves the addition of a methyl group to the 5 position of the cytosine, and different histone modifications.

DNA methylation at a gene promoter reveals a repressive chromatin environment that typically does not allow gene expression, while DNA demethylation allows it. This event mostly occurs in CpG dinucleotides, and is catalyzed by *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b*, and maintained by *Dnmt1* (Bird 2002). Some important mechanisms regulated by DNA methylation are genomic imprinting and X chromosome inactivation.

1.1. Genomic imprinting

Genomic imprinting is a mechanism that allows that a subset of genes are expressed only from the paternally or from the maternally inherited allele, unlike most genes in the genome, that are expressed or silenced from both alleles. This mechanism is regulated by DNA methylation dynamics in differentially methylated regions (DMRs) within the genome (Arnaud 2010).

Genomic imprints must be erased and reestablished during the gametogenesis so they can be transmitted to the next generation in a sex-specific manner. Thus, epigenetic reprogramming takes place in primordial germ cells (PGCs) shortly after they reach the gonadal ridges carrying the allele-specific imprints characteristic of somatic cells (Surani 2001). Imprints erasure through active demethylation starts by 10.5 dpc, and is completed by 13.5 dpc in the mouse (Hajkova, Erhardt et al. 2002, Yamazaki, Mann et al. 2003). However, imprints must be reestablished before the onset of meiosis. In the female germline, this happens in growing oocytes after birth, during meiotic prophase I (Hajkova, Erhardt et al. 2002, Allegrucci, Thurston et al. 2005), and in the male germline imprints are reestablished in early gonocytes during fetal development (Lucifero, Mertineit et al. 2002) (Figure 3). Disruptions in this epigenetic reprogramming may cause several growth and behavioral syndromes (Clayton-Smith 2003, Chen, Robbins et al. 2013).

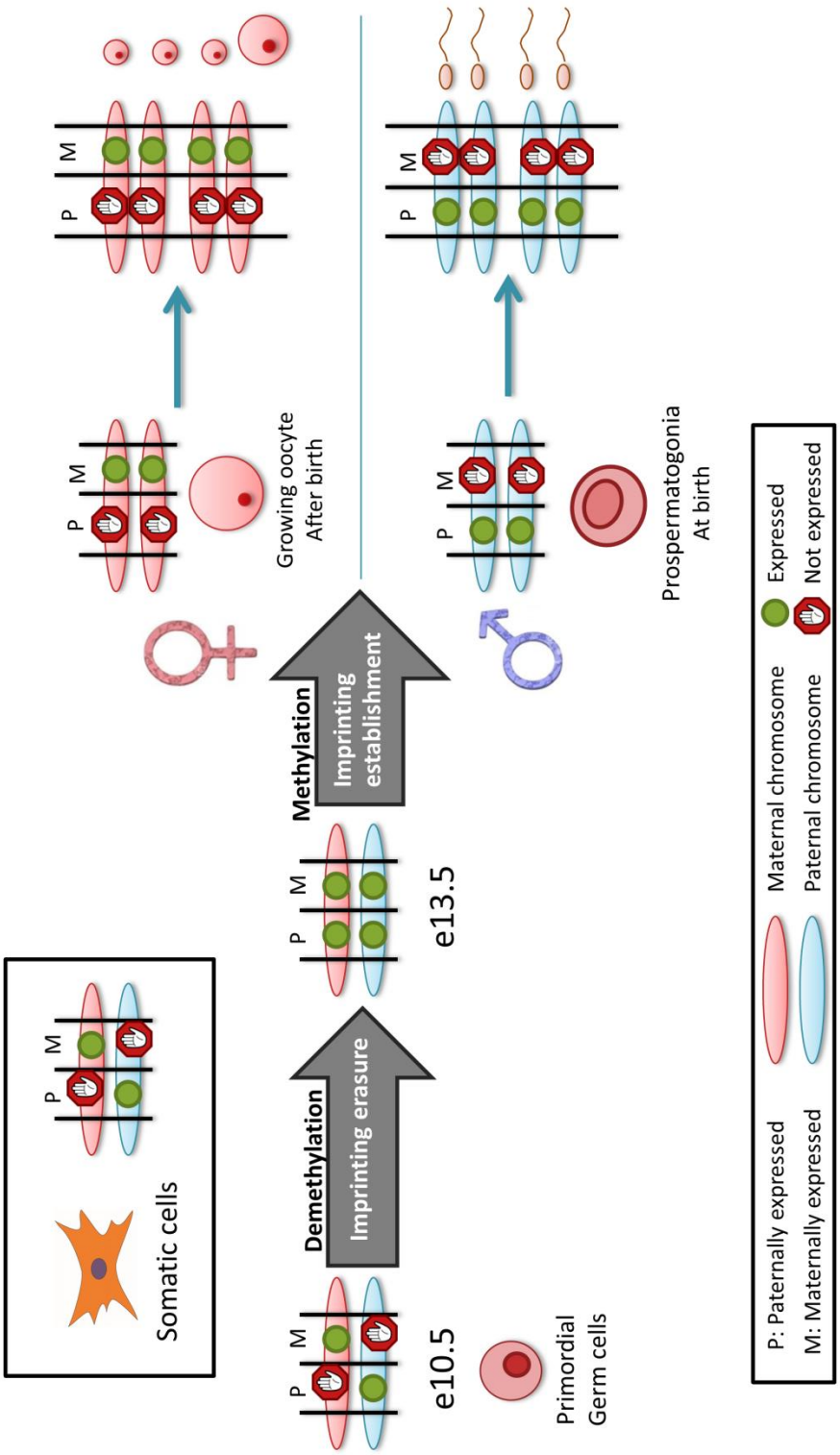

Figure 3. Genomic imprinting reprogramming

1.2. X chromosome inactivation (XCI)

X-chromosome inactivation (XCI) is a complex epigenetic mechanism that is required to ensure that most X-linked genes are expressed equally in both sexes (Lyon 1961). The process compensates the double dosage of X-linked genes by the transcriptional silencing of one of the X chromosomes in females. This silencing occurs randomly in the maternal or paternal inherited X chromosome early in embryogenesis, while the extra-embryonic tissues inactivate only the paternal X chromosome in mice (Bermejo-Alvarez, Ramos-Ibeas et al. 2012).

XCI is initiated by the expression of the RNA *Xist* (X-inactive specific transcript), which recruits several proteins that coat the X chromosome by diverse epigenetic marks. The most characteristic is the trimethylation of histone H3 at lysine 27 (H3K27me3), a reppresive mark that impedes transcription (Bermejo-Alvarez, Ramos-Ibeas et al. 2012).

In the early epiblast of the blastocyst, both X chromosomes are active in females. Thus, it would be expected that ESCs derived from these epiblasts show an absence of XCI. However, human ESCs are highly heterogeneous in their XCI status, and presence of both X chromosomes in an active state has been proposed as a hallmark of ground-state pluripotency and a quality marker for female ESCs. In the same way, XCI reversal in female somatic cell reprogramming is a key event to achieve naïve pluripotency (Bermejo-Alvarez, Ramos-Ibeas et al. 2012).

2. Influence of embryonic source on ESCs epigenetics and pluripotency

ESCs lines can be derived from embryos that may have different characteristics, and in some cases these characteristics, or even alterations, can be transmitted to their corresponding ESCs lines.

Disease-carrying human ESCs have been obtained from affected embryos following preimplantation genetic diagnosis. In this way, hESC lines were derived carrying mutations for myotonic dystrophy type 1, cystic fibrosis, Huntington´s disease (Mateizel, De Temmerman et al. 2006), adrenoleukodystrophy, Duchenne and Becker muscular dystrophy and thalassaemia (Verlinsky, Strelchenko et al. 2005). These cell lines provide a powerful *in vitro* tool for modelling disease progression, identifying molecular mechanisms that may prevent this progression, and investigating drugs *in vitro* toxicity and efficacy (Stephenson, Mason et al. 2009). In the same way, human iPSC lines have been obtained from patients with a variety of neurological diseases and used to produce a number of neuronal subtypes (Juopperi, Song et al. 2011).

However, in other circumstances, embryonic characteristics are not reflected by their derived ESCs lines. Before iPSCs were first derived, somatic cell nuclear transfer (SCNT) allowed for the derivation of ESCs lines from somatic cells of diseased individuals that could be differentiated into a host of cell types for cell replacement therapy. In the beginning, it was rejected as a valid technology in humans because of the severely abnormal phenotypes observed in tissues of cloned animals (Brambrink, Hochedlinger et al. 2006). However, it was later demonstrated that these abnormal phenotypes were not carried by SCNT-derived ESCs lines, as SCNT- and fertilization-derived ESCs lines were functionally and transcriptionally indistinguishable. In contrast to SCNT-derived animals, the process of SCNT-ESCs lines derivation could select for those cells that have erased the "epigenetic memory" of the donor nucleus (Brambrink, Hochedlinger et al. 2006, Ding, Guo et al. 2009).

ESCs lines derivation involves the manipulation and *in vitro* culture of blastocysts. These embryos are epigenetically dynamic and very sensitive to environmental variations, so epigenetic alterations can be induced by their manipulation and culture in non-physiological conditions, which can persist during adulthood and cause disorders such as obesity, infertility and behavior and growth alterations (Ramirez, Pericuesta et al. 2006, Fernandez-Gonzalez, Moreira et al. 2008, Calle, Miranda et al. 2012).

It has been demonstrated that *in vitro* embryo culture can produce imprinting alterations, resulting in biallelic expression of some genes as *H19*, *Igf2* and *Igf2r*; and these alterations are transmitted to the ESC lines. In some cases, they can be corrected by continuous culture (Ramirez, Pericuesta et al. 2007, Horii, Yanagisawa et al. 2010); however, some studies indicate that they persist after differentiation producing aberrant genetic expression patterns (Feinberg, Ohlsson et al. 2006). Most of the imprinted genes regulate growth and cell proliferation, so their epigenetic deregulation could have oncogenic effects (Morison, Ramsay et al. 2005). Actually, chimeric mice generated from ESCs with global loss of imprinting develop multiple tumors (Holm, Jackson-Grusby et al. 2005).
Especially worrying is the case of human ESCs, because all cell lines are derived from embryos donated by patients undergoing assisted reproductive techniques (ARTs), and are believed to be unsuitable for use or cryopreservation (Stephenson, Mason et al. 2009). In the last years, it has been reported a significantly increased risk of birth defects in infants conceived by ARTs, although possibly this increased risk may be due to the underlying infertility of the couples pursuing ARTs, and not to ARTs themselves (Wen, Jiang et al. 2012, Wen, Jiang et al. 2012, Hansen, Kurinczuk et al. 2013, Vermeiden and Bernardus 2013). Abnormalities found in ART-produced embryos could be maintained in ESCs lines derived from them, as it was demonstrated by Horii *et al.*, who derived ESCs lines from and *in vitro* fertilized (IVF)-embryos, and although derivation efficiency was not significantly different to *in vivo*-produced embryos, they observed abnormal genomic

imprinting and expression patterns of methylation-related genes in IVF-derived ESCs lines at early passages. In contrast, there was no significant difference at later passages (Horii, Yanagisawa et al. 2010).

Intracytoplasmic sperm injection (ICSI) is currently the most commonly used method to overcome male infertility; however, some studies have suggested that ICSI bypasses natural selection barriers, allowing sperm with damaged DNA to fertilize an oocyte (Schultz and Williams 2002, Hourcade, Perez-Crespo et al. 2010). Furthermore, 40% of the infertile men following ICSI treatment have high proportions of DNA strand breaks or other types of DNA damage in their sperm (Lopes, Jurisicova et al. 1998, Sergerie, Laforest et al. 2005, Zini, Meriano et al. 2005). In mice, it has been reported that embryos produced by ICSI using DNA-fragmented sperm are genetically and epigenetically altered. However, some of them implant and develop into animals that show aberrant growth, premature ageing, abnormal behavior, and mesenchymal tumors (Fernandez-Gonzalez, Moreira et al. 2008). Consequently, ICSI using DNA fragmented sperm could be used as a model to analyze if ESCs lines derived from these embryos display the same alterations observed in the animals, and if pluripotency is affected. In this way, the generation of animals for the study of ARTs secondary effects could be avoided.

3. Influence of the cellular source on iPSCs epigenetics and pluripotency

After the discovery of induced pluripotency, some reports suggested that the reprogrammed iPSCs retained some epigenetic memory of the cell type of origin, as they maintained the expression of some transcripts characteristisc from the original cell, and this was associated with variability in their differentiation capacity (Kim, Doi et al. 2010, Polo, Liu et al. 2010). Kim *et al*. compared SCNT-derived ESCs, *in vivo* fertilized embryo-derived ESCs and low passage iPSCs derived from fibroblasts or blood. They observed an intriguing difference in differentiation propensities; while both SCNT- and *in vivo* embryo-derived ESCs could differentiate readily down every lineage, iPSCs preferentially differentiated towards a specific lineage linked to their cell of origin; the blood lineage and the osteogenic pathway. DNA methylation analysis in DMRs showed that whereas SCNT- and *in vivo* embryo-derived ESCs were very similar, iPSC lines were dissimilar to each other and differed to ESCs (Kim, Doi et al. 2010).

Stadtfeld *et al*. realized that some iPSCs lines that lacked the full development potential of ESCs also showed silencing of some imprinted genes. They showed that a few transcripts encoded within the imprinted *Dlk1-Dio3* cluster were aberrantly silenced in iPSCs clones that contributed poorly to chimaeras and failed to support the development

of entirely iPSC-derived animals by tetraploid complementation assay. However, this locus could be reactivated just by treatment with a histone deacetylase inhibitor, rescuing the ability to support full-term development of all-iPSC mice (Stadtfeld, Apostolou et al. 2010).

Polo *et al*. showed that the differences among iPSCs derived from different somatic cell types became more obvious when epigenetic analysis was extended to histone modifications. Excitingly, this study demonstrates that continuous passaging of iPSCs leads to the erasure of the differences observed, as early passage (passage 4) iPSCs were different transcriptionally, epigenetically and on their differentiation potential; but by passage 16, these differences were abrogated. However, the observed silencing of the imprinted *Dlk1-Dio3* cluster was not modified by passaging of cells, suggesting that not all epigenetic modifications are reset (Polo, Liu et al. 2010).

Influence of culture conditions on pluripotency

1. Influence of culture conditions on pluripotency capture *in vitro*

Despite the fact that culture conditions shown in literature are not always properly detailed, and that ESCs-specific markers are limited and have not been described for most species, it is remarkable that there are more similitudes than differences in culture conditions used for ESCs derivation in different vertebrates.

Different cell types have been used as feeder layers for ESCs culture in diverse species, including homologous and heterologous embryonic fibroblasts (EFs). Homologous EFs have been successfully employed for ESCs derivation in mink, marsupial, human and porcine. In species as sheep, cow and chicken, the use of homologous EFs has failed to support ESCs (Familari and Selwood 2006). It is possible that EFs in these species are not able to produce the essential factors for self-renewal, or perhaps the gestational stage in which these feeder layers were obtained was not equivalent to the functional stage in which murine EFs are derived (mid-gestation). Some of the heterologous feeder layers employed for ESCs derivation are Buffalo rat liver cells in mice (Smith and Hooper 1987); bovine umbilical cord cells in equine (Saito, Ugai et al. 2002); human fetal lung fibroblasts in cow (Gjorret and Maddox-Hyttel 2005); and rainbow trout spleen fibroblasts in zebrafish (Fan, Crodian et al. 2004). However, murine EFs or STO (a transformed murine fibroblasts cell line) allow for the derivation of ESCs in most mammals and are nowadays used.

Mouse ESCs grow as round compact colonies of small cells, which depend on the LIF/STAT3 pathway. Traditionally, culture conditions consisted on a “feeder” cell layer of mitotically inactivated mouse EFs, and medium supplemented with foetal calf serum (FCS) and leukemia inhibitory factor (LIF) (Evans and Kaufman 1981). LIF is a cytokine produced by the endometrium, which allows blastocyst implantation (Pera and Tam 2010). In ESCs, LIF binds to Gp130 receptor and maintains self-renewal and pluripotency by phosphorylating STAT3 (Williams, Hilton et al. 1988, Niwa, Burdon et al. 1998). The effect of LIF on the *in vitro* development of embryos has been widely studied but results are often contradictory. Some studies have demonstrated that LIF has the capacity to enhance blastocyst formation and to decrease embryo fragmentation in mouse (Tsai, Chang et al. 1999, Cheng, Huang et al. 2004), while other studies have shown the opposite results that LIF in standard medium does not enhance the development of early embryos in human and in bovine (Jurisicova, Ben-Chetrit et al. 1995, Gutierrez-Adan, Perez-Crespo et al. 2006). These contradictory results may be attributable to the different species and culture systems used for the experiments. Nevertheless, LIF appears to be a unifying property of both ESCs maintenance and blastocyst diapause. This common LIF-dependence has been a persistent argument for a diapause-like state of naïve ESCs, and this is further supported by the fact that entering diapause increases the efficiency of ESCs derivation from blastocysts (Brook and Gardner 1997, Welling and Geijsen 2013). Thus, it is interesting to investigate whether or not the embryos cultured *in vitro* supplemented with LIF are more suitable for ESCs derivation.

Later, an innovative culture system for ESCs derivation and culture was implemented in which mitogen-activated protein kinase (MAPK/MEK) signaling was eliminated and glycogen synthase kinase 3 (GSK3) activity was reduced (Ying, Wray et al. 2008). This culture system combines LIF with two kinase inhibitors (2i): PD0325901, a MEK inhibitor that inhibits the mitogen-activated protein kinase enzymes MEK 1 and MEK2, and CHIR99021, a GSK3β inhibitor, in a defined medium without the use of FCS. The LIF-2i system allows efficient derivation and expansion of germline-competent ESCs from different strains of mice that were previously recalcitrant for ESCs derivation (Ying, Wray et al. 2008, Nichols, Jones et al. 2009) and, for the first time, from the rat (Buehr, Meek et al. 2008, Li, Tong et al. 2008, Ying, Wray et al. 2008, Leitch, Blair et al. 2010). Moreover, 2i supplementation during embryo culture suppresses the formation of the hypoblast, resulting in the entire ICM becoming pluripotent epiblast, and enhancing ESCs derivation efficiency (Nichols, Silva et al. 2009). Furthermore, reprogramming efficiency of mouse somatic cells to iPSCs can be enhanced as well by 2i supplementation (Silva, Barrandon et al. 2008).

Different growth factors have been used to supplement culture medium in order to isolate pluripotent cells that are remarkably similar to ESCs; Embryonic Germ Cells (EGCs)

have been isolated from Primordial Germ Cells (PGCs) by LIF, stem cell factor (SCF) and basic fibroblast growth factor (bFGF) supplementation (Resnick, Bixler et al. 1992). In the same way, pluripotent Germline Stem Cells (GSCs) have been isolated by gonocyte or Spermatogonial Stem Cells (SSCs) *in vitro* culture in the presence of LIF, bFGF, Epidermal Growth Factor (EGF) and Glial cell-derived neurotrophic factor (GDNF) (Kanatsu-Shinohara, Ogonuki et al. 2003, Kanatsu-Shinohara, Inoue et al. 2004). These factors also affect the proliferation and maintenance of other stem cell populations: EGF is a powerful mitogen which enhances tissue regeneration in various adult organs such as skin, liver and intestinal epithelium (Reynolds and Weiss 1992); and GDNF has been shown to stimulate SSC self-renewal *in vivo* (Meng, Lindahl et al. 2000).

Interestingly, recent studies have described that the pluripotent cell transcriptome is defined by culture conditions, and not by embryonic origin or derivation procedure. Thus, major differences in gene expression were observed when pluripotent cells were cultured in medium with serum *vs*. 2i conditions, while slight differences were observed between ESCs coming from blastocysts *vs*. EGCs coming from PGCs, cultured in the same conditions (Marks, Kalkan et al. 2012, Leitch, McEwen et al. 2013).

Consequently, it would be interesting to analyze the process of ESCs derivation in different culture conditions.

2. Influence of culture conditions on epigenetics and pluripotency maintenance

Once established, ESCs lines can be maintained in culture for unlimited time; however, pluripotency can be lost (Nagy, Gocza et al. 1990) and epigenetic alterations as methylation modifications in imprinted genes can appear (Fujimoto, Mitalipov et al. 2005, Rugg-Gunn, Ferguson-Smith et al. 2005, Horii, Yanagisawa et al. 2010). In human ESCs, it has been described that long term culture affects the methylation of the promoter region of tumor suppressor genes (Maitra, Arking et al. 2005). Other studies indicate that retroviral DNA introduced in ESCs, which is normally silenced by methylation, can be reactivated by loss of methylation caused by culture conditions (Allegrucci, Wu et al. 2007, Minoguchi and Iba 2008). These changes are unpredictable and variable among different cell lines, and once established they can be stable and be transmitted to differentiated cells (Lund, Narva et al. 2012). However, until now optimal culture conditions for epigenetic stability remain unknown.

In the early epiblast in female blastocysts, both X chromosomes are still in an active state, so it would be expected the same situation for ESCs lines derived from these embryos. Nevertheless, high variability has been found among cell lines, especially in human ESCs

(Bermejo-Alvarez, Ramos-Ibeas et al. 2012). Culture conditions as oxygen concentration can affect XCI. Hypoxic conditions promote that both X chromosomes are active in female cell lines, while changes from hypoxic to normoxic conditions promote X inactivation (Lengner, Gimelbrant et al. 2010). Likewise, cellular stress such as freeze-thaw cycles makes the cells more prone to X inactivation (Lund, Narva et al. 2012). Furthermore, some XX ESC lines show a global loss of DNA methylation, possibly associated to the fact that both X chromosomes are in an active state. Consequently, XX cell lines are more unstable in culture than XY, so generally in research XY cell lines are more preferable (Zvetkova, Apedaile et al. 2005, Pannetier and Feil 2007).

Pluripotency in adult tissues

In the last years, it has been described the existence of adult stem cells (ASCs) that support multiple tissues. These cells are multipotent, meaning that they are limited to differentiate towards a specific cell lineage. ASCs can be identified in many tissues and organs, including skeletal muscle, bone, cartilage, skin, blood vessels, heart, liver, gut, lung, brain, fat tissue, dental pulp, amniotic fluid, peripheral blood, ovarian epithelium, testis and bone marrow (Macaluso and Myburgh 2012). They reside in specific areas in the organs called “niches”; specialized local microenvironments where they are protected and supported while they maintain a low basal rate of division. These niches comprise specific extracellular matrix and supporting cells, and have the potential to modulate signaling towards stem cell self-renewal or differentiation (Greco and Guo 2010).

During lifetime, a number of ASCs will be activated in order to maintain tissues homeostasis, while the remaining ASCs will stay in a quiescent phase; both coexisting in the same area (Li and Clevers 2010). Their location and their slowly proliferating state make them much more difficult to study than other pluripotent cells as ESCs (Alvarez, Garcia-Lavandeira et al. 2012). However, nowadays numerous subtypes of ASCs have been described: haematopoietic and mesenchymal stem cells residing in bone marrow; gut stem cells located in the crypts of Lieberhahn, liver stem cells, bone and cartilage stem cells, skin and hair stem cells, neuronal stem cells, pancreatic stem cells, retinal stem cells, cardiac stem cells, dental pulp stem cells and skeletal muscle stem cells (Schabort, Myburgh et al. 2009). Much research has been done on the regenerative potential of these ASCs in animal models of tissue damage. Similarly, their ability to transdifferentiate *in vitro* has been investigated with a view to *ex vivo* cell expansion and manipulation for transplant (Macaluso and Myburgh 2012). After purification, ASCs can be grown *in vitro* with selected growth factors, micronutrients, antioxidants and

hormones supplementation at supposedly physiological concentrations (Alvarez, Garcia-Lavandeira et al. 2012).

Probably one of the most studied ASCs subtypes is spermatogonial stem cells (SSCs). SSCs are able to undergo self-renewal division yielding new SSCs, or to differentiate towards spermatogonias to support spermatogenesis (Kanatsu-Shinohara, Inoue et al. 2011). Although the estimated number of SSCs in the mouse testis comprises only 0.03% of the total germ cell population (de Rooij and Mizrak 2008), techniques have been developed that allow isolation and long-term culture of SSCs giving rise to a population called Germline Stem cells (GSCs). Moreover, it has been described the appearance of ES-like colonies called multipotent Germline Stem cells (mGSCs) after long-term culture (Kanatsu-Shinohara, Ogonuki et al. 2003, Kanatsu-Shinohara, Inoue et al. 2004, Guan, Nayernia et al. 2006).

Although common ESCs markers, such as *Oct4*, *Sox2*, *Nanog*, *Klf4*, *Lin28* or *Fut4* (*Ssea1)* are expressed in some ASCs as hematopoietic stem cells (HSCs) (Wang, Zhou et al. 2010), mesenchymal stem cells (MSCs) (Jaramillo-Ferrada, Wolvetang et al. 2012) and SSCs (Izadyar, Pau et al. 2008), ASCs also express specific markers depending on their niche location. However, good markers common to all ASCs or organ-specific are not yet well known, because some of them are also expressed by differentiated cells.

Pluripotency and germline common markers

1. Germline specification and development

Germline specification is one of the earliest cell fate decisions made in the mammalian embryo. PGCs induction starts prior to gastrulation in the proximal epiblast at embryonic day (e) 6.25 due to the bone morphogenetic proteins (BMPs) stimulation, mainly by *Bmp4* and to a lesser extent to *Bmp8b*, that are released from the extraembryonic ectoderm (ExE) (Lawson, Dunn et al. 1999, Ying, Qi et al. 2001). These factors activate the ACVR1 (*Alk2*) serine-threonine kinase receptor (de Sousa Lopes, Roelen et al. 2004), which induces phosphorylation and nuclear translocation of SMAD1, SMAD5 and SMAD8 transcription factors, enabling modulation of target genes expression (de Sousa Lopes, Hayashi et al. 2007). These events direct a small group of 4-8 cells to enter the germinal lineage by *Blimp1* and *Prdm14* upregulation, the two crucial determinants of the germline (Ohinata, Payer et al. 2005, Saitou and Yamaji 2012). These cells proliferate and develop a cluster of 20 cells that subsequently express other PGC markers such as *Fragilis* (*Ifitm3*) and *Stella* (*Dppa3*) by e7.5. The pluripotency-associated transcription factors *Pou5f1 (Oct4)* and *Nanog* are initially expressed in the ICM but become restricted to the germline between e7.75 and 8.5 (Young, Dias et al. 2010). Thereafter, PGCs initiate migration and colonization of the genital ridge and increase their number to approximately 4000 by e12.5 (Saitou, Barton et al. 2002, Sato, Kimura et al. 2002). Once PGCs reach the gonads, other germline-specific markers essential for the development of the germ cells are transcribed, including *Ddx4* (Toyooka, Tsunekawa et al. 2000) and *Dazl* (Lin and Page 2005).

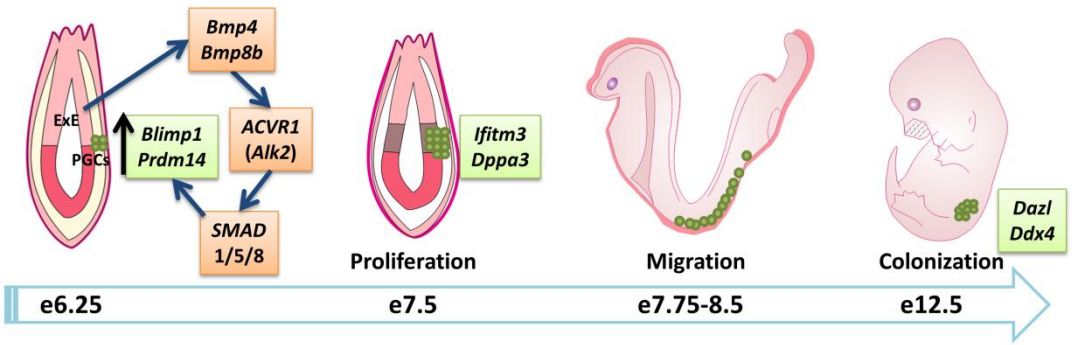

Figure 4. PGC specification and development

2. Expression of germline markers in pluripotent cells

There are different pluripotent cell types according to the source of cells used for their establishment: ESCs derived from early epiblasts, EGCs from PGCs, ECCs isolated from germ-cell tumors, GSCs derived from SSCs, and iPSCs from somatic cells reprogramming (Xu, Pantakani et al. 2011). Most of the above mentioned have a germ-cell origin apart from ESCs and iPSCs, but all of them share common germ cell or premeiotic markers that may indicate a common germinal origin (Zwaka and Thomson 2005) (Table 1).

Interestingly, *Oct4, Sox2* and *Nanog*, key pluripotency genes, appear to be necessary for germ cell survival (Kehler, Tolkunova et al. 2004, Yamaguchi, Kimura et al. 2005, Chambers, Silva et al. 2007, Zhao, Ji et al. 2012). Furthermore, several studies have demonstrated that germline or premeiotic markers as *Blimp1*, *Dppa3*, *Ifitm3*, *Piwil2*, *Dazl*, *Ddx4*, *Stra8*, *Rnf17* and *Rnh2* are expressed in ESCs (Geijsen, Horoschak et al. 2004, Qing, Shi et al. 2007, Mise, Fuchikami et al. 2008, Xu, Pantakani et al. 2011). Moreover, a recent study associates *Dazl* with Tet (Ten eleven translocation)-dependent DNA demethylation in ESCs (Blaschke, Ebata et al. 2013). Another common marker for pluripotency and germline is *Prdm14*, whose essential role is confined to the germline establishment (Yamaji, Seki et al. 2008), although it has been recently shown to play a role in epigenetic regulation ESCs to achieve naïve pluripotency (Yamaji, Ueda et al. 2013).

In the same way, these germline and premeiotic markers, and even meiotic markers *Sycp3*, *Pgk2*, and *Creb3/4*, were detected also in iPSC, GSCs and EGCs and in both male and female cell lines. When somatic cell reprogramming process was analyzed, germ cell markers *Blimp1, Dppa3* and *Ifitm3* started to be expressed between day 6 and 9 of reprogramming, and by day 22 their expression increased to the levels observed in ESCs. In contrast, significant expression of the key pluripotency markers *Pou5f1* and *Sox2* started later, at day 12. Other premeiotic markers as *Stra8*, *Dazl* and *Ddx4* were only detected after day 22 of reprogramming (Xu, Pantakani et al. 2011).

Furthermore, it has been recently described a novel alternatively spliced isoform of *mDazl* with a deletion of exon8, referred to as *Dazl_Δ8*, that is expressed together with the full-length isoform of *Dazl* in different pluripotent cell types: ESCs, GSCs, iPSCs , EGCs and ECCs (Xu, Tan et al. 2013).

In mouse preimplantation embryos, germline markers *Dppa3*, *Dazl* and *Ddx4* have been detected throughout all stages (Pan, Liao et al. 2008, Xu, Pantakani et al. 2011). In non-mammal species, *zDazl* has been localized in early zebrafish embryos (Maegawa, Yasuda et al. 1999, Hashimoto, Maegawa et al. 2004) and *XDazl* in Xenopus embryos until stage 10 (Houston and King 2000).

Concerning adult stem cells, it has been described *DAZL*, *c-Kit* and *POU5F1* expression in human amniotic fluid stem cells (hAFSCs) (Stefanidis, Loutradis et al. 2008). Furthermore, *Pou5f1*, *Dppa3*, *Ifitm3*, *Dazl* and *Ddx4* have been located in mouse and human bone

marrow (BM) (Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005). Another study described that mouse bone marrow mesenchymal stem cells (BM-MSCs) were positive for *Dazl*, but in lower levels of expression than the total bone marrow cells tested (Katsara, Mahaira et al. 2011).

Table 1. Pluripotency and germline common markers

Molecular marker	Expression	References
Fut4 (Ssea1)	ESC, PGC, EGC, ECC, iPSC, embryo	(Knowles, Aden et al. 1978, Solter and Knowles 1978, Fox, Damjanov et al. 1981, Zhao, Ji et al. 2012)
c-Kit	ESC, PGC, ECC, HSC	(Zhao, Ji et al. 2012)
UTF1	ESC, ECC, ICM, germ cells	(Kooistra, Thummer et al. 2009, Zhao, Ji et al. 2012)
Pou5f1	ESC, PGC, EGC, ECC, iPSC, ICM, bone marrow	(Pesce and Scholer 2000, Pesce and Scholer 2001, Kehler, Tolkunova et al. 2004, Zhao, Ji et al. 2012)
Dppa3	ESC, PGC, ECC, iPSC, embryo, bone marrow	(Bowles, Teasdale et al. 2003, Benson, Karsch-Mizrachi et al. 2004, Bortvin, Goodheart et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Xu, Pantakani et al. 2011, Zhao, Ji et al. 2012)
Sox2	ESC, PGC, EGC, ECC, iPSC, embryo	(Botquin, Hess et al. 1998, Boyer, Lee et al. 2005, Zhao, Ji et al. 2012)
GCNF	ESC, ECC, germ cells	(Lei, Hirose et al. 1997, Lan, Xu et al. 2009, Zhao, Ji et al. 2012)
Nanog	ESC, PGC, EGC, ECC, iPSC	(Chambers, Silva et al. 2007, Yamaguchi, Kurimoto et al. 2009)
Prdm14	ESC, PGC	(Yamaji, Seki et al. 2008, Leitch, McEwen et al. 2013, Yamaji, Ueda et al. 2013)
Blimp1	ESC, PGC, iPSC	(Xu, Pantakani et al. 2011)
Ifitm3	ESC, PGC, iPSC, bone marrow	(Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Xu, Pantakani et al. 2011)
Piwil2	ESC, PGC,	(Xu, Pantakani et al. 2011)
Dazl	ESC, PGC, EGC, GSC, ECC, iPSC, embryo, bone marrow	(Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Pan, Liao et al. 2008, Katsara, Mahaira et al. 2011, Xu, Pantakani et al. 2011, Xu, Tan et al. 2013)

Ddx4	ESC, PGC embryo, iPSC, bone marrow	(Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Xu, Pantakani et al. 2011)
Stra8	ESC, PGC, iPSC	(Xu, Pantakani et al. 2011)
Rnf17	ESC, PGC	(Xu, Pantakani et al. 2011)
Rnh2	ESC, PGC	(Xu, Pantakani et al. 2011)

ESC: embryonic stem cell; PGC: primordial germ cell; EGC: embryonic germ cell; ECC: embryonic carcinoma cell; iPSC: induced pluripotent stem cell; GSC: germline stem cell; HSC: hematopoietic stem cell; ICM: inner cell mass.

In conclusion, germ cell and premeiotic markers have been found to be expressed in numerous pluripotent cell types, which may indicate a common germ cell origin for them. According to this theory, the blastocyst stage (e3.5) expresses key pluripotency markers as *Pou5f1* and *Sox2*. Following embryonic development, PGC specification takes place when *Blimp1* expression is activated, repressing the somatic program and reactivating the pluripotency network before PGC migration (Saitou 2009). In the same way, ESCs derivation starts with e3.5 blastocysts culture, and cells would proceed with the developmental program of PGCs specification via BMPs signaling and would begin expressing *Blimp1*, *Ifitm3* and *Dppa3* among other cell markers, and reactivating pluripotency genes to acquire self-renewal properties. Similarly, during somatic cell reprogramming by *Pou5f1*, *Sox2*, *c-Myc* and *Klf4* factors, inductive BMPs signaling would trigger germ cell markers expression and finally reactivate pluripotency network (Xu, Pantakani et al. 2011) (Figure 5).

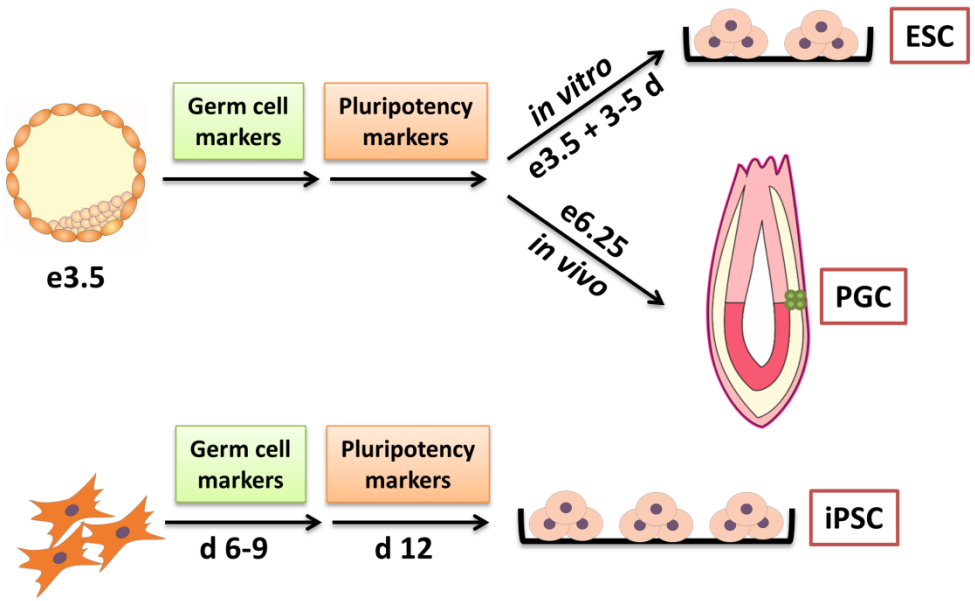

Figure 5. Theory of the germ cell origin of pluripotent cells

Transgenic mice where a reporter gene expression as green fluorescen protein (GFP) is driven by a known gene promoter constitute an ideal model to study gene localization in several tissues along different developmental periods, as not only allows easy identification of cells expressing the transgene, but also the reporter gene does not interact with biological processes. *Dazl* is one of the genes which main function is related to germline development and meiosis (Ruggiu, Speed et al. 1997), although some recent studies have related *Dazl* to pluripotency (Haston, Tung et al. 2009, Xu, Tan et al. 2013).

Trophectoderm specification and pluripotency

The first tissue lineages segregation occurs when blastocyst cells differentiate into both the inner cell mass (ICM), which will give rise to the embryo proper, and the trophectoderm (TE), the precursor of the placenta (Schiffmacher and Keefer 2013). Both lineages are controlled by gene regulatory networks, but specie-specific differences exist.

1. Specie-specific differences in trophectoderm specification

In mice, there is an antagonistic genetic interaction between ICM and TE regulators. The ICM and its associated pluripotent state are maintained by *Pou5f1* (*Oct4*), while TE specification relies on *Cdx2*, which is able to suppress *Pou5f1* expression and *vice versa*. However, this situation varies markedly between embryos from different mammalian species. *Pou5f1* expression is restricted to the ICM in mice, but in cattle and porcine embryos it is expressed in the TE as well, so the mouse embryo is not an ideal model for ungulate embryogenesis (Wolf, Serup et al. 2011). It has been suggested that the early down-regulation of *Pou5f1* in rodents could have evolved to allow a rapid commitment to the TE fate and a shorter preimplantation development, while in cattle and other mammals where preimplantation development is longer, there may not be such need (Berg, Smith et al. 2011).

The precursors of the differentiated cells of the placenta are assumed to be trophoblast stem cells (TSCs). They are supposed to exist in all placental mammals, especially during the early stages of placental development when TE growth is maximal. However, little is known about their location and phenotype in other species than mouse and rhesus macaque (Roberts and Fisher 2011). In the mouse, the TSCs niche appears to be located within the extraembryonic ectoderm (ExE) adjacent to the epiblast, on which depends for essential growth factors. This specialized niche is transient and maintained only for 3 or 4 days during postimplantation development. Mouse TSCs can be obtained from proliferating outgrowths of polar TE explanted from a region bordering the epiblast and cultured *in vitro* on mitotically inactivated feeder cells and a medium supplemented with FBS, Fibroblast Growth Factor 4 (FGF4) and heparin (Tanaka, Kunath et al. 1998). Cells with some properties of TSCs have been obtained from rhesus macaque blastocysts as well (Vandevoort, Thirkill et al. 2007).

Domestic species such as swine, sheep and cattle show a dramatic elongation of the TE preceeding implantation that allows to exploit uterine secretions throughout a long area of the uterine lumen (Bindon 1971, Geisert, Brookbank et al. 1982, Thatcher, Meyer et al. 1995). Unlike mouse, polar TE overlying the embryonic disk is soon lost in these species and does not contribute to the placenta, while the homolog of mouse mural TE is

responsible of the elongation. It is unknown whether the expansion of mural TE relies on a stem cell population immediately surrounding the embryonic disk, or on stem cells scattered throughout the TE; or whether sustained growth is entirely due to proliferation of progenitor cells that are already partially differentiated (Roberts and Fisher 2011). Consequently, TSCs have not been isolated from any ungulate species, although primary trophectoderm cell lines have been derived from sheep and goat (Miyazaki, Imai et al. 2002), pig (Ramsoondar, Christopherson et al. 1993, Flechon, Laurie et al. 1995, La Bonnardiere, Flechon et al. 2002) and cattle (Talbot, Caperna et al. 2000, Shimada, Nakano et al. 2001, Suzuki, Koshi et al. 2011).

2. Bovine trophoblast cell lines derivation

Trophoblast cells grow *in vitro* as a tight monolayer that eventually forms dome-like structures. These structures accumulate fluid until they transform into spheroids that are released to the culture medium.

In bovine, trophoblast cell lines were first derived by Talbot *et al.* from the culture of 7-8-days *in vitro*-produced blastocysts over STO feeder cells (Talbot, Caperna et al. 2000). However, the co-culture with mouse feeder cells has the risk of contaminating the bovine trophoblastic cells, and this could be detrimental for using these cell lines for further studies. Later, Shimada *et al.* (Shimada, Nakano et al. 2001, Hirata, Sato et al. 2003) established a bovine trophoblastic cell line in the absence of feeder cells, using bovine endometrial fibroblast-conditioned medium, but attaining very low derivation efficiency. They plated more than 50 blastocysts, obtaining only one cell line. The mayor problem appeared to be the attachment of the blastocyst to the culture plate and the initial proliferation. Since then, most of the studies regarding trophectoderm signaling and differentiation have been performed with these only two trophoblastic cell lines derived by Talbot and Shimada (Nakano, Shimada et al. 2002, Hirata, Sato et al. 2003, Ushizawa, Takahashi et al. 2005, Michael, Wagner et al. 2006, Das, Ezashi et al. 2008, Nakaya, Kizaki et al. 2009, Bai, Sakurai et al. 2011, Schiffmacher and Keefer 2012, Schiffmacher and Keefer 2013). Therefore, it would be helpful to come with a larger number of trophoblastic cell lines acceptable for *in vitro* studies, without any possible cell contamination.

Trophoblastic cell lines have been derived from parthenogenetic and SCNT-produced embryos, and significant differences have been found in derivation efficiency, as parthenogenetic blastocysts had a lower ratio for primary colony formation than *in vivo* or SCNT-produced blastocysts. In the same way, the amount of the pregnancy establishment-protein Interferon tau (INF-t) produced by outgrowths obtained from parthenogenetic and SCNT-produced embryos was lower than in those dericed from *in*

vitro fertilized embryos. Furthermore, SCNT-derived trophoblastic cell lines had less annexin proteins, related to placentation maintenance (Talbot, Powell et al. 2007, Talbot, Powell et al. 2008, Talbot, Powell et al. 2010). Therefore, trophoblastic cell lines could reflect the alterations carried by the embryos, and could be used as a model to analyze them.

In a more recent study, Suzuki *et al.* used BMP4 to raise trophoblastic cell lines based on the fact that exogenous BMP4 stimulate ESCs to become trophoblastic cells (Murohashi, Nakamura et al. 2010), but no significant improvement was found in derivation efficiency, and just 12 trophoblastic cell lines were derived from 172 embryos. When genes necessary for pregnancy establishment, interferon-t (*IFNT*), placental lactogen (*CSH1*), prolactin-related protein 1 (*PRP1*) and pregnancy-associated glycoprotein 1 (*PAG1*), were analyzed, very variable expression patterns were found among the different cell lines (Suzuki, Koshi et al. 2011). Thus, there is a high variability among trophoblastic cell lines obtained using the same derivation conditions. Many of the derived trophoblastic cell lines grow continuously lacking senescence symptoms, and show pluripotency marker *POU5F1* expression (Suzuki, Koshi et al. 2011, Schiffmacher and Keefer 2013). Therefore, they could represent the pluripotent cell population from which the placenta differentiates in cattle, or a more differentiated equivalent to mouse TSCs.

Trophoblastic cell lines represent a useful model for implantation and placentogenesis studies, and they could reflect genetic and epigenetic characteristics and alterations carried by the embryos from which they are derived. However, just a small number of cell lines are used for research due to the difficulty of establishing new cell lines. Consequently, it would be interesting to establish an efficient system for trophoblastic cell lines derivation from a TE biopsy that would allow the embryo to be viable, while its characteristics or alterations are analyzed on its corresponding trophoblastic cell line.

Introducción

El descubrimiento de la pluripotencia

1. Del descubrimiento de las células pluripotentes a la captura de la pluripotencia *in vitro*

La pluripotencia y las células troncales han fascinado a biólogos y médicos desde hace más de un siglo.

El término "célula troncal" fue empleado por primera vez en la literatura científica en 1868 por el ilustre biólogo alemán Ernst Haeckel, que utilizó el término "Stammzelle" para describir el organismo unicelular a partir del cual todos los organismos multicelulares evolucionaron (Haeckel 1868). Posteriormente, propuso referirse también al huevo fertilizado como "Stammzelle" (Haeckel 1877). Por tanto, según Haeckel, el término célula troncal podría ser usado de dos maneras: como el antepasado unicelular común a todos los organismos multicelulares, y como el huevo fertilizado que da lugar a todas las células del organismo (Ramalho-Santos and Willenbring 2007). En 1892, otro científico alemán, Theodor Boveri, llevó la definición de célula troncal como huevo fertilizado un paso más allá: propuso que el término fuese usado para las primeras células de linaje germinal que aparecen en el embrión animal, que presuntamente transportan el plasma germinal y posteriormente se diferencian para formar los gametos (Boveri 1892, Ramalho-Santos and Willenbring 2007). Por lo tanto, en estos primeros estudios, el término célula troncal se refería a lo que actualmente llamamos el linaje germinal, o células germinales primordiales.

Cuatro años después, el término fue popularizado en su versión inglesa por Edmund B. Wilson, un científico americano que revisó los estudios de Boveri en su famoso libro *The Cell in Development and Inheritance* (Wilson 1896). Este libro inspiró a todos los embriólogos y genetistas de la época, y a Wilson se le adjudicó el mérito de haber acuñado el término.

En la misma época, se generó un gran interés científico en el desarrollo y la regeneración del sistema hematopoyético, y un grupo de científicos empezó a creer en la existencia de una célula que representara el origen común de diferentes tipos celulares de la sangre. Alguno de ellos empezó a usar el término célula troncal para referirse a dicho precursor común (Pappenheim 1896, Ramalho-Santos and Willenbring 2007).

Por tanto, las primeras interpretaciones del término célula troncal a finales del siglo XIX se referían a cuestiones fundamentales en embriología: la continuidad de la línea germinal y el origen del sistema sanguíneo.

Posteriormente, la existencia de las células troncales hematopoyéticas ("Hematopoietic stem cells", HSCs) quedó demostrada (Till and McCulloch 1961, Becker, Mc et al. 1963,

Till, McCulloch et al. 1964) y estas células fueron consideradas como el prototipo de célula troncal, capaz de proliferar indefinidamente (autorrenovación) y de dar lugar a células más especializadas (diferenciación) (Figura 1).

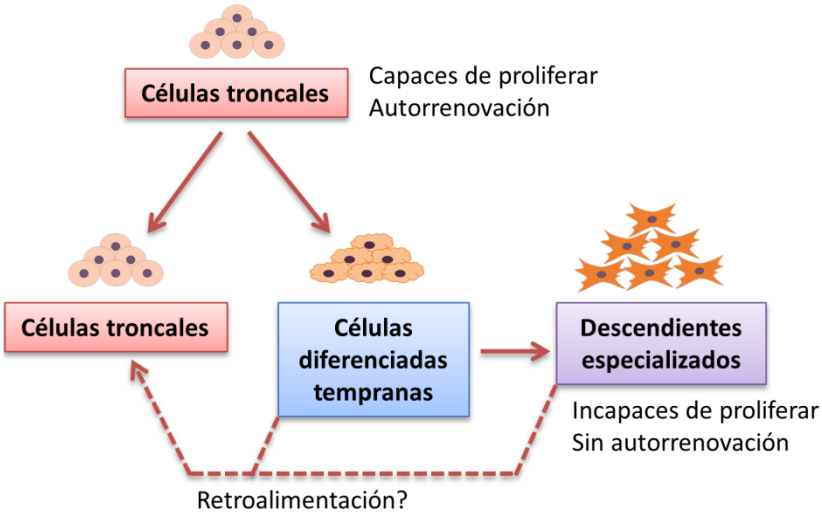

Figura 1. Representación de las vías de proliferación de las células precursoras de la sangre (Till, McCulloch et al. 1964).

Las bases del cultivo de células pluripotentes fueron establecidas gracias a la investigación con teratocarcinomas murinos. El descubrimiento de que los machos de la cepa murina 129 tenían una alta incidencia de teratocarcinomas testiculares, y de que el crecimiento de dichos teratocarcinomas dependía de las células embrionarias de carcinoma ("Embryonal carcinoma cells", ECCs) (Stevens and Little 1954) marcó el inicio de la experimentación con tumores, que se llevaba a cabo mediante su trasplante en serie entre ratones. Posteriormente se establecieron las condiciones de cultivo para las ECCs, que fueron utilizadas como un modelo *in vitro* de desarrollo en mamíferos (Kahan and Ephrussi 1970). Además, se observó que el trasplante ectópico de anillos germinales y de embriones preimplantacionales también inducía la formación de teratocarcinomas; es decir, estos se debían al trasplante de fuentes de células pluripotentes (Stevens 1967).

El siguiente paso fue intentar producir células pluripotentes directamente *in vitro,* y se consiguió al poner en cultivo blastocistos tardíos con las condiciones de cultivo previamente establecidas para el cultivo de las ECCs. Estas fueron las primeras células troncales embrionarias ("Embryonic stem cells", ESCs) que se aislaron (Evans and Kaufman 1981). Se comprobó que, a diferencia de las ECCs, las ESCs eran capaces de producir animales quiméricos no sólo en el soma, sino contribuyendo también a la línea germinal y así a su descendencia, por lo que resultaron ser unas herramientas muy

eficaces para la manipulación genética (Bradley, Evans et al. 1984, Robertson, Bradley et al. 1986) (revisado en (Solter 2006)).

En 1992 se aislaron células pluripotentes *in vitro* a partir de una fuente diferente, las células germinales primordiales ("Primordial germ cells", PGCs), mediante el uso de factores de crecimiento como suplemento para el medio de cultivo convencional de ESCs (Matsui, Zsebo et al. 1992). Estas células compartían importantes características con las ESCs, incluyendo su morfología, pluripotencia y capacidad de transmisión a línea germinal en animales quiméricos. Fueron llamadas células germinales embrionarias ("Embryonic germ cells", EGCs), para distinguirlas de las ESCs derivadas a partir de blastocistos.

Desde que las ESCs fueron aisladas por primera vez en el ratón, pasaron diecisiete años hasta que se aislaron las primeras ESCs humanas (Thomson, Itskovitz-Eldor et al. 1998). Esta demora probablemente fue debida a las diferencias existentes entre el ratón y el humano a nivel de desarrollo embrionario. Además, la pluripotencia de estas células es menor a la de sus equivalentes murinas, pues no son capaces de generar quimeras en sus condiciones de cultivo estándar (recientemente se ha descubierto que utilizando ciertos factores en el medio de cultivo se puede incrementar su pluripotencia, haciendo así que se asemejen a las ESCs murinas y que sean capaces de generar quimeras (Gafni, Weinberger et al. 2013)). Además, las ESCs humanas forman colonias de morfología plana, asemejándose a las líneas celulares derivadas a partir de epiblastos tardíos murinos, llamadas células troncales del epiblasto (EpiSCs) (Tesar, Chenoweth et al. 2007); a diferencia de las ESCs murinas, que forman colonias tridimensionales. Por lo tanto, se han propuesto dos tipos de pluripotencia: la **naïve**, donde se incluyen las ESCs murinas que dependen de la vía LIF/Stat3, y la **primed**, donde se incluyen las ESCs de primates y ungulados y las EpiSCs murinas que dependen de la señalización de Fgf/ERK (Nichols and Smith 2009).

Aunque el aislamiento de ESCs humanas fue reconocido como un gran avance médico, generó conflictos éticos respecto a la utilización de embriones humanos para investigación. Estos inconvenientes fueron superados gracias al descubrimiento de los factores de reprogramación (*Oct4*, *Sox2*, *Klf4*, *c-Myc*) por Yamanaka *et al*., que permitieron la reprogramación de células somáticas a células pluripotentes, dando lugar a las células de pluripotencia inducida ("induced pluripotent stem cells", iPSCs) por primera vez en ratón (Takahashi and Yamanaka 2006), y posteriormente en humanos omitiendo *cMyc* y usando *LIN28* en su lugar (Takahashi, Tanabe et al. 2007).

En resumen, en las últimas décadas se han obtenido diferentes tipos de células pluripotentes en diferentes momentos del desarrollo que pueden ser cultivadas *in vitro* a la vez que mantienen su autorrenovación y pluripotencia (Figura 2).

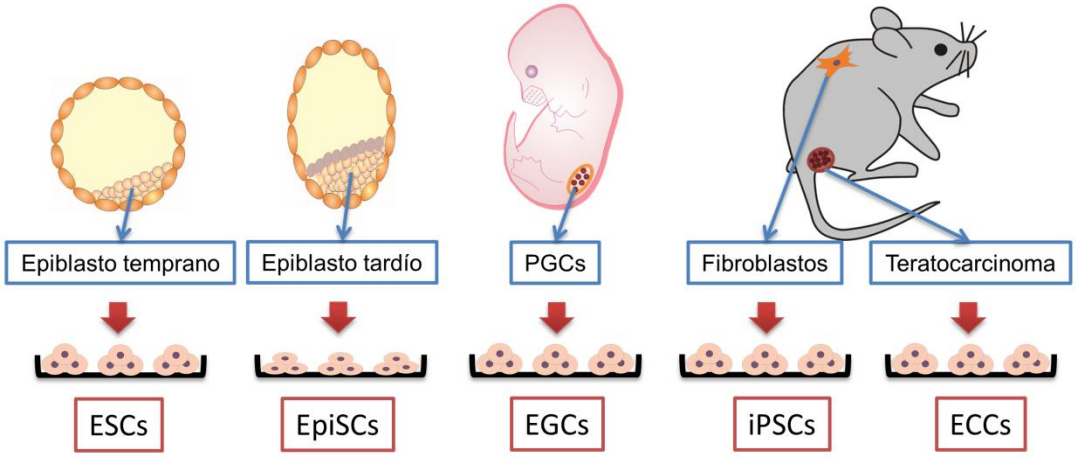

Figura 2. Tipos de células pluripotentes. Durante el desarrollo preimplantacional pueden aislarse ESCs a partir de epiblastos tempranos de blastocistos tardíos. A partir de epiblastos tardíos de embriones recién implantados se aíslan EpiSCs. Posteriormente, las EGCs pueden obtenerse a partir de PGCs de fetos, y durante la edad adulta pueden obtenerse iPSCs a través de la reprogramación de células somáticas y ECCs a partir de teratocarcinomas.

2. Diferentes teorías sobre el origen de las ESCs

A lo largo de su desarrollo, un cigoto totipotente da lugar a billones, incluso trillones de células diferenciadas de manera coordinada para formar un organismo (Niwa and Fujimori 2010).

La evolución del embrión en vertebrados da lugar por un lado a linajes extraembrionarios y por otro lado a células pro-embrionarias. Las células pro-embrionarias se caracterizan por ser células pluripotentes; es decir, mantienen un estado de no diferenciación, y presentan una alta capacidad mitótica (Johnson 2001), lo que conduce a su capacidad de formar ESCs. Estas células reciben distintas denominaciones en función del grupo taxonómico al que hagamos referencia y en función del estadio de desarrollo. En el ratón se les denomina masa celular interna (“inner cell mass”, ICM) y más tarde epiblasto (Rossant 2001). En aves reciben el nombre de germen, después blastodermo y a continuación epiblasto (Eyal-Giladi and Kochav 1976). En marsupiales constituyen el pluriblasto y después el epiblasto (Johnson and Selwood 1996).

La morfología y la distribución de los linajes celulares (células pro-embrionarias y TE) también varía según la taxonomía. En marsupiales, tanto pluriblasto como TE son superficiales conformando un blastocisto unilaminar. Tras la diferenciación del hipoblasto se forma un blastocisto bilaminar, en el cual tanto el epiblasto como el TE son superficiales. En las aves, el estadio X de Hamburger y Hamilton del embrión de pollo

consta de dos regiones visiblemente diferenciadas: el *area pellucida*, a partir del cual se desarrolla el embrión, y la zona opaca circundante (*area opaca*) que da lugar al saco vitelino extraembrionario (Eyal-Giladi and Kochav 1976). Es también en el estadio X, donde comienza a formarse el hipoblasto, de modo que en el estadio XI, el *area pellucida* comprende tanto epiblasto como hipoblasto. Por ello, el estadio X de desarrollo es el empleado en aves para el aislamiento de ESCs y es también el momento para realizar *gene targeting* y generar animales quiméricos. En el pez cebra, el pez medaka y la dorada, tras la décima división (mid-blástula), el genoma del cigoto se activa dando lugar a los primeros tres linajes celulares. Dos de ellos son linajes extra-embrionarios: la capa sincitial de la yema, y la capa externa que la rodea, y la tercera capa es el pluriblasto, que se encuentra en el interior (Fan, Crodian et al. 2004). Para el aislamiento de ESCs en estas tres especies de peces se ha empleado el estadio de mid-blástula, y también el anillo germinal en el caso del pez cebra.

En el ratón, a día 3,5 de desarrollo embrionario, el blastocisto comprende dos linajes celulares, la ICM y el trofoectodermo ("trophectoderm", TE). La ICM da lugar al ectodermo primitivo o epiblasto, mientras que el trofoblasto sólo contribuye al tejido placentario (Rossant 2001). Tras la diferenciación del hipoblasto (endodermo primitivo), la ICM residual se convierte en el epiblasto temprano post-implantacional, y estas células pluripotentes darán lugar rápidamente a las hojas germinales primarias durante la gastrulación. Históricamente se ha asumido que las ESCs murinas eran equivalentes a las células de la ICM, debido a que el aislamiento de las ESCs se consiguió gracias el cultivo de blastocistos de día 3,5. Sin embargo, cada vez hay más indicios de que las ESCs tienen un origen diferente. Hace unos años surgió una teoría que suponía la existencia de una subpoblación celular del epiblasto que era seleccionada durante el proceso de aislamiento y daba lugar a las ESCs; y un candidato interesante para esta subpoblación eran las células predispuestas a diferenciarse hacia el linaje germinal (Zwaka and Thomson 2005). Apoyando esta teoría, por una parte las PGCs son capaces de generar líneas celulares pluripotentes que son idénticas a las ESCs (Matsui, Zsebo et al. 1992, Resnick, Bixler et al. 1992), y por otra parte, de todos los linajes celulares que se diferencian a partir del epiblasto, sólo las células germinales expresan genes relacionados con la pluripotencia como *Oct4*, *Nanog* y *Sox2* (Durcova-Hills and Surani 2008, Chu, Surani et al. 2011). Además, se ha observado que el factor de especificación del linaje germinal *Blimp1* y otros marcadores de células germinales como *Dppa3 (Stella)* y *Prdm14* se activan cuando se ponen en cultivo blastocistos para aislar ESCs en medio suplementado con suero fetal bovino ("fetal calf serum"; FCS) y LIF (Chu, Surani et al. 2011), lo que sugiere que las células que van a dar lugar a las ESCs activan transitoriamente un programa transcripcional específico de PGCs (Hochedlinger 2011). De hecho, cuando estas células que sobreexpresan *Blimp1* son aisladas y trasplantadas a embriones

deficientes en células germinales a día 8,5, migran a los anillos germinales y comienzan a sobreexpresar el marcador de desarrollo de linaje germinal *Mvh*. Además, aislando las células que sobreexpresan *Blimp1* se pudieron obtener ESCs con una eficiencia nueve veces mayor que con todo el cúmulo de células de la ICM. Sin embargo, este estado transitorio de célula germinal no parece ser estrictamente necesario para la obtención de ESCs, ya que las ESCs pueden aislarse a partir de blastocistos knockout que no expresan *Blimp1* (Chu, Surani et al. 2011, Nichols and Smith 2011). Por otra parte, la obtención de ESCs con el sistema 2i (explicado más adelante en la introducción) no provoca sobreexpresión de *Blimp1*, sino que permite capturar directamente el potencial de auto-renovación de las células del epiblasto sin pasar por un programa germinal transitorio (Hochedlinger 2011).

Señales y pruebas de pluripotencia

Las células pluripotentes se caracterizan por su capacidad de dividirse indefinidamente a la vez que mantienen un estado indiferenciado o auto-renovación, y por su potencial para diferenciarse en cualquiera de las tres hojas embrionarias (endodermo, mesodermo y ectodermo).

Existen diferentes técnicas *in vivo* e *in vitro* para analizar la pluripotencia de una población celular. Las células pluripotentes "naïve" crecen *in vitro* como colonias tridimensionales de bordes redondeados, mientras que la aparición de colonias planas es característica de células pluripotentes "primed" o puede ser señal de diferenciación (Nichols, Silva et al. 2009, Nichols and Smith 2009).

Uno de los protocolos más utilizados para detectar pluripotencia es la tinción de la Fosfatasa Alcalina, ya que las células pluripotentes indiferenciadas tienen unos niveles elevados de esta enzima. Por otra parte, existe un amplio panel de marcadores moleculares y bioquímicos específicos de células pluripotentes, que son fundamentales para mantener su estado indiferenciado. En el ratón, los principales marcadores de pluripotencia son *Oct4*, *Nanog*, *Sox2* y *Fut4 (Ssea1)* (Marti, Mulero et al. 2013).

Asimismo pueden llevarse a cabo estudios de diferenciación *in vivo* e *in vitro*. Las células pluripotentes pueden diferenciarse *in vitro* formando cuerpos embrioides (Embryoid bodies, EBs); agregados celulares redondos y compactos que crecen en suspensión generando las tres hojas embrionarias (endodermo, mesodermo y ectodermo). De la misma manera, cuando las células pluripotentes son inyectadas en lugares ectópicos en animales receptores, producen teratomas que contienen diferentes tejidos diferenciados representativos de las tres hojas embrionarias *in vivo*. Curiosamente, no sólo las células

totalmente pluripotentes se diferencian formando las tres hojas embrionarias, ya que las ESCs humanas y las EpiSCs murinas también son capaces de formar EBs y teratomas (Alvarez, Garcia-Lavandeira et al. 2012).

La prueba más importante para asegurar que una población celular es pluripotente es la generación de animales quiméricos combinando embriones receptores con células pluripotentes, que sean capaces de transmitir la información genética procedente de las células pluripotentes al linaje germinal. Uno de los métodos para generar estas quimeras es el ensayo de complementación con tetraploides. Los embriones tetraploides pueden generarse fusionando las células de un embrión en estadio de dos células mediante una corriente eléctrica. Estos embriones pueden desarrollarse de manera normal hasta el estadio de blastocisto, y las células tetraploides son capaces de formar los tejidos extraembrionarios; sin embargo, raramente son capaces de formar el feto. En el ensayo de complementación con tetraploides, un embrión tetraploide en estadio de mórula o de blastocisto se combina con células pluripotentes diploides. De esta manera el embrión es capaz de desarrollarse de manera normal porque procede exclusivamente de las células pluripotentes, mientras que los tejidos extraembrionarios procederán de las células tetraploides. Otro método para generar animales quiméricos es la microinyección de células pluripotentes en un embrión receptor en estadio de mórula o de blastocisto (Ramirez, Fernandez-Gonzalez et al. 2009) o la agregación de células pluripotentes con un embrión en estadio de 8 blastómeras (Ramirez, Pericuesta et al. 2007). Sólo las células verdaderamente pluripotentes serán capaces de integrarse en el blastocisto receptor y de contribuir a la formación de todos los órganos del animal, y en especial del linaje germinal para permitir la transmisión de la información genética a las siguientes generaciones, y esto es característico de las células pluripotentes en estado naïve. Por el contrario, las células pluripotentes en estado primed, como las EpiSCs murinas y las ESCs en especies como el cerdo, el bovino y el humano, son incapaces de contribuir a la formación de animales quiméricos, lo que demuestra que no son completamente pluripotentes (Brevini, Antonini et al. 2008, Alvarez, Garcia-Lavandeira et al. 2012). Sin embargo, en algunos casos se ha conseguido obtener animales quiméricos a partir de células pluripotentes en estado naïve en el cerdo (Fujishiro, Nakano et al. 2013), en humanos (Gafni, Weinberger et al. 2013) y en la oveja (Sartori, DiDomenico et al. 2012).

Influencia de la fuente embrionaria o celular en la epigenética y la pluripotencia de las ESCs

1. Modificaciones epigenéticas

Las modificaciones epigenéticas son todos aquellos cambios que puede sufrir la cromatina que modifican la expresión génica sin que se vea afectada la secuencia de nucleótidos. Esto se consigue principalmente mediante dos mecanismos: la metilación del ADN, que consiste en la adición de un grupo metilo en el carbono 5 de las citosinas, y las modificaciones en las histonas. La metilación del ADN en la región promotora de un gen supone una marca represiva sobre la cromatina que normalmente no permite que dicho gen se exprese; mientras que la falta de metilación permite su expresión.

La metilación del ADN tiene lugar principalmente en los dinucleótidos CG, y está catalizada por las ADN metil-transferasas (*Dnmt*), siendo *Dnmt1* la responsable del mantenimiento de la metilación, y *Dnmt3a* y *Dnmt3b* las encargadas de la metilación *de novo*. Algunos de los mecanismos más importantes regulados epigenéticamente son la impronta genómica y la inactivación del cromosoma X.

1.1. La impronta genómica

La impronta genómica es un mecanismo que permite que determinados genes se expresen sólo a partir del alelo heredado de la madre o del alelo heredado del padre, a diferencia de la mayoría de los genes del genoma, que se expresan a partir de ambos alelos. Este mecanismo está regulado por la metilación del ADN en las regiones diferencialmente metiladas ("Differentially methylated regions", DMRs) del genoma (Arnaud 2010).

Las marcas de imprinting deben ser borradas y reestablecidas durante la gametogénesis para que puedan ser transmitidas a la siguiente generación de manera específica en función del sexo. Para ello, durante el desarrollo fetal tiene lugar una reprogramación epigenética que permite borrar estas marcas de metilación en las PGCs (Surani 2001). El borrado de las marcas de imprinting mediante desmetilación activa comienza en el feto del ratón a día 10,5 y se completa a día 13,5 (Hajkova, Erhardt et al. 2002, Yamazaki, Mann et al. 2003). Sin embargo, las marcas de imprinting deben ser restablecidas antes del comienzo de la meiosis. En el linaje germinal femenino se reestablecen en los ovocitos en desarrollo tras el nacimiento, durante la profase I de la meiosis (Hajkova, Erhardt et al. 2002, Allegrucci, Thurston et al. 2005), y en el masculino en los gonocitos durante el desarrollo fetal (Lucifero, Mertineit et al. 2002) (Figura 3). Las alteraciones en la

reprogramación de la impronta genómica pueden producir síndromes relacionados con el crecimiento y el comportamiento (Clayton-Smith 2003, Chen, Robbins et al. 2013).

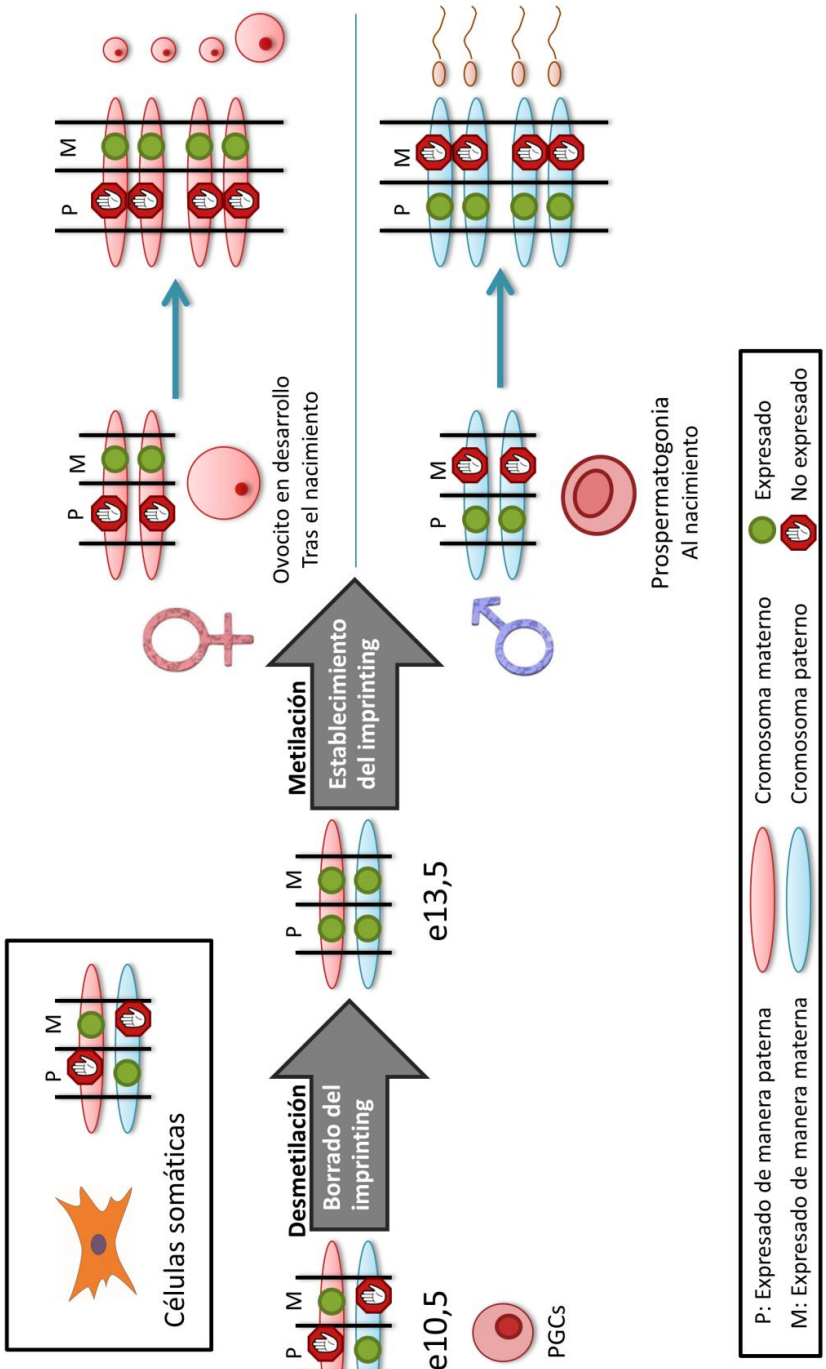

Figura 3. Reprogramación de la impronta genómica

1.2. La inactivación del cromosoma X

La inactivación del cromosoma X es otro complejo mecanismo regulado epigenéticamente que asegura que la mayoría de los genes localizados en el cromosoma X se expresen de igual manera en ambos sexos (Lyon 1961). Este proceso compensa la doble dosis de genes localizados en el cromosoma X mediante el silenciamiento de uno de ellos en las hembras. Este silenciamiento tiene lugar en estadios tempranos de la embriogénesis en el ratón; y en el embrión se produce al azar en el cromosoma X heredado de la madre o en el heredado del padre, mientras que en los tejidos extraembrionarios sólo se inactiva el cromosoma X paterno (Bermejo-Alvarez, Ramos-Ibeas et al. 2012).

La inactivación del cromosoma X se inicia por la expresión del ARN *Xist* (X-inactive specific transcript), que recluta diferentes proteínas que recubren el cromosoma X mediante diferentes marcas epigenéticas. La más característica es la trimetilación de la histona H3 en la lisina 27 (H3K27me3), una marca represiva que impide la transcripción génica (Bermejo-Alvarez, Ramos-Ibeas et al. 2012).

En el epiblasto temprano del blastocisto, ambos cromosomas X están activos en el caso de las hembras. Por ello, cabría esperar que las ESCs obtenidas a partir de estos epiblastos mostrasen una ausencia de inactivación del cromosoma X. Sin embargo, las ESCs humanas son muy heterogéneas en su estado de inactivación del cromosoma X, por lo que se ha propuesto que el hecho de que ambos cromosomas X se encuentren activos es un indicador de calidad y de verdadera pluripotencia en las líneas de ESCs hembras. De la misma manera, durante la reprogramación de células somáticas, la reactivación del cromosoma X es un evento clave para conseguir un estado de pluripotencia naïve (Bermejo-Alvarez, Ramos-Ibeas et al. 2012).

2. Influencia de las características del embrión en la epigenética y la pluripotencia de las ESCs

Las ESCs pueden obtenerse a partir de embriones de diferentes características, y en algunos casos dichas características, o incluso alteraciones, pueden transmitirse a sus correspondientes ESCs.

El diagnóstico genético preimplantacional ha permitido el aislamiento de ESCs humanas portadoras de enfermedades a partir de embriones afectados. De esta manera se han obtenido ESCs portadoras de mutaciones responsables de distrofia miotónica tipo 1, fibrosis quística, enfermedad de Huntington (Mateizel, De Temmerman et al. 2006), adrenoleucodistrofia, distrofia muscular de Duchenne y de Becker y talasemia (Verlinsky, Strelchenko et al. 2005). Estas líneas celulares suponen una poderosa herramienta para estudiar la progresión de estas enfermedades, identificar mecanismos moleculares que

puedan frenar esta progresión, y hacer estudios de toxicidad y de eficacia de fármacos *in vitro* (Stephenson, Mason et al. 2009). De la misma manera, se han obtenido iPSC humanas a partir de pacientes con diferentes enfermedades neurológicas, y se han conseguido diferenciar para dar lugar a diferentes subtipos neuronales (Juopperi, Song et al. 2011).

Sin embargo, en otras circunstancias, las características del embrión no se ven reflejadas en las líneas de ESCs derivadas a partir de ellos. Antes del descubrimiento de las iPSCs, se obtuvieron líneas de ESCs a partir de células somáticas de individuos portadores de enfermedades gracias a la transferencia nuclear de células somáticas ("Somatic Cell Nuclear Transfer", SCNT), que podían ser diferenciadas hacia multitud de tipos celulares y ser usadas para medicina regenerativa. Al principio, esta técnica fue rechazada para su uso en humanos debido a las graves anormalidades observadas en animales clonados (Brambrink, Hochedlinger et al. 2006). Sin embargo, posteriormente se demostró que dichas anormalidades no se transmitían a las ESCs obtenidas a través de SCNT, ya que varios estudios demostraron que estas ESCs eran funcional y transcripcionalmente idénticas a las ESCs derivadas a partir de embriones fertilizados de manera natural. A diferencia de lo que ocurre en los animales producidos mediante SCNT, el proceso de obtención de las ESCs podría seleccionar sólo aquellas células que hubieran borrado la "memoria epigenética" del núcleo de la célula donante (Brambrink, Hochedlinger et al. 2006, Ding, Guo et al. 2009).

La obtención de líneas de ESCs implica la manipulación y cultivo *in vitro* de blastocistos. Estos embriones se encuentran en un estadio de desarrollo epigenéticamente dinámico y muy sensible a los cambios ambientales, y al ser manipulados y sometidos a condiciones de cultivo no fisiológicas, pueden producirse alteraciones epigenéticas que persistan durante la edad adulta y que se traduzcan en problemas de salud como obesidad, infertilidad o alteraciones del comportamiento y del crecimiento (Ramirez, Pericuesta et al. 2006, Fernandez-Gonzalez, Moreira et al. 2008, Calle, Miranda et al. 2012).

Se ha demostrado que el cultivo *in vitro* de los embriones puede producir alteraciones en la impronta genómica, resultando en la expresión bialélica de algunos genes como *H19*, *Igf2* e *Igf2r*. Estas alteraciones se transmiten a las líneas de ESCs, y en algunos casos el cultivo continuado puede hacer que se corrijan (Ramirez, Pericuesta et al. 2007, Horii, Yanagisawa et al. 2010); sin embargo, otros estudios indican que se mantienen tras la diferenciación y dan lugar a patrones de expresión génica aberrantes (Feinberg, Ohlsson et al. 2006). Muchos de los genes improntados se encargan de regular el crecimiento y la proliferación celular, por lo que su alteración podría dar lugar a la aparición de tumores (Morison, Ramsay et al. 2005). De hecho, se ha comprobado que los ratones quiméricos

generados a partir de ESCs con pérdida global de impronta genómica desarrollan múltiples tumores (Holm, Jackson-Grusby et al. 2005).

Esto es especialmente preocupante en humanos, donde debido a la legislación vigente y a cuestiones éticas, todas las líneas de ESCs se generan a partir de embriones producidos *in vitro* en las clínicas de infertilidad y donados al no ser apropiados para ser transferidos o congelados. (Stephenson, Mason et al. 2009). En los últimos años se ha descrito un incremento significativo en el riesgo de padecer ciertos síndromes en los niños concebidos mediante técnicas de reproducción asistida ("Assisted reproductive technologies", ARTs), aunque es posible que este riesgo se deba a la infertilidad subyacente de las parejas que se someten a ARTs, y no a las técnicas en sí (Wen, Jiang et al. 2012, Hansen, Kurinczuk et al. 2013, Vermeiden and Bernardus 2013). Las anormalidades observadas en los embriones producidos mediante ARTs podrían transmitirse a las líneas de ESCs obtenidas a partir de ellos, como fue demostrado por Horii *et al*., quienes obtuvieron líneas de ESCs a partir de embriones producidos por fertilización *in vitro* ("*in vitro* fertilization", IVF); y aunque la eficiencia de obtención fue similar a la de embriones producidos *in vivo*, pudieron observar alteraciones en la impronta genómica y en los patrones de expresión de genes relacionados con la metilación en las líneas de ESCs de embriones producidos por IVF en pases tempranos. Por el contrario, estas diferencias desaparecían en pases posteriores (Horii, Yanagisawa et al. 2010).

La inyección intracitoplasmática de espermatozoides ("Intracytoplasmic sperm injection", ICSI) es la técnica más utilizada actualmente en los casos de infertilidad masculina; sin embargo, algunos estudios sugieren que la ICSI se salta barreras de selección naturales, permitiendo que los ovocitos sean fertilizados por espermatozoides con daños en el ADN (Schultz and Williams 2002, Hourcade, Perez-Crespo et al. 2010). Además, el 40% de los hombres infértiles que se someten a tratamiento con ICSI tienen una alta proporción de roturas de hebras en el ADN u otros tipos de daño en el ADN de su esperma (Lopes, Jurisicova et al. 1998, Sergerie, Laforest et al. 2005, Zini, Meriano et al. 2005). En el ratón se ha descrito que los embriones producidos por ICSI utilizando esperma con ADN fragmentado muestran alteraciones genéticas y epigenéticas. A pesar de ello, algunos son capaces de implantarse y de dar lugar a animales que muestran crecimiento aberrante, envejecimiento prematuro, comportamiento anormal y tumores mesenquimales (Fernandez-Gonzalez, Moreira et al. 2008). Por ello, la ICSI utilizando esperma con ADN fragmentado podría servir como un modelo para analizar si las líneas de ESCs obtenidas a partir de estos embriones reflejan las alteraciones observadas en los animales, y si su pluripotencia se ve afectada. De esta manera se evitaría tener que generar animales para el estudio del impacto de las ARTs.

3. Influencia de las características de la célula de origen en la epigenética y la pluripotencia de las iPSCs

Tras el descubrimiento de la pluripotencia inducida, algunos estudios han sugerido que las iPSCs retienen cierta memoria epigenética de la célula de la que proceden, ya que mantienen la expresión de algunos transcritos de la célula original. Esto se ha asociado con cierta variabilidad entre líneas en cuanto a su capacidad de diferenciación (Kim, Doi et al. 2010, Polo, Liu et al. 2010). Kim *et al.*, hicieron un estudio comparativo entre líneas de ESCs derivadas a partir de embriones producidos mediante SCNT y fertilización *in vivo*, y líneas de iPSCs producidas a partir de fibroblastos o células sanguíneas; todas ellas con bajo número de pases. Observaron diferentes tendencias de diferenciación entre las líneas celulares: mientras que todas las líneas de ESCs eran capaces de diferenciarse fácilmente hacia cualquier linaje celular, las iPSCs mostraban una tendencia a diferenciarse hacia ciertos linajes relacionados con su célula de origen; es decir, hacia los linajes sanguíneo u óseo. Tras un análisis de las DMRs se vio que las líneas de ESCs derivadas a partir de embriones producidos mediante SCNT y fertilización *in vivo* eran muy similares, pero que las líneas de iPSCs eran diferentes a las ESCs, y a su vez unas de otras (Kim, Doi et al. 2010).

Stadtfeld *et al.* también descubrieron que algunas líneas de iPSCs no tenían el mismo potencial que las ESCs, y que esto estaba relacionado con silenciamiento del locus *Dlk1-Dio3*. Demostraron que algunos transcritos codificados por el cluster improntado *Dlk1-Dio3* estaban aberrantemente silenciados en aquellos clones de iPSCs que contribuían pobremente en la generación de quimeras, y que eran incapaces de mantener el desarrollo de animales completamente generados a partir de iPSCs mediante el ensayo de complementación con tetraploides. Sin embargo, este locus pudo ser reactivado mediante el tratamiento con un inhibidor de deacetilasas de histonas, rescatando así la capacidad de generar ratones íntegramente a partir de iPSCs que llegasen a término (Stadtfeld, Apostolou et al. 2010).

Polo *et al*. señalaron que las diferencias entre líneas de iPSCs generadas a partir de diferentes tipos celulares eran más obvias cuando los análisis epigenéticos se extendían a las modificaciones de histonas. Además, su estudio demuestra que cuando las iPSCs son mantenidas en cultivo, estas diferencias se borran a lo largo de repetidos pases; ya que en un pase temprano (pase 4), las líneas de iPSCs mostraban diferencias a nivel transcripcional, epigenético y en cuanto a potencial de diferenciación; pero en el pase 16 estas diferencias se eliminaban. Sin embargo, el silenciamiento del cluster *Dlk1-Dio3* no se veía modificado por el número de pases, lo que sugiere que no todas las modificaciones epigenéticas pueden ser restauradas (Polo, Liu et al. 2010).

Influencia de las condiciones de cultivo en la epigenética y en la pluripotencia

1. Influencia de las condiciones de cultivo en la captura de la pluripotencia *in vitro*

Aunque los detalles de las condiciones de cultivo mostrados en la literatura no siempre son suficientes, y los patrones de expresión de marcadores de ESCs no son extensos o no se han descrito para la mayoría de las especies, existen más similitudes que diferencias en las condiciones de cultivo utilizadas para obtener ESCs en diferentes especies de vertebrados.

Se han probado muchos tipos celulares homólogos y heterólogos como "feeder layers" para el cultivo de ESCs, incluyendo fibroblastos embrionarios ("embryonic fibroblasts", EFs) murinos. Los EFs homólogos han sido empleados con éxito para obtener ESCs en visón, marsupial, humano y porcino. En otros casos como la oveja, la vaca y el pollo, el uso de EFs homólogos no ha sido adecuado (Familari and Selwood 2006). Esto puede sugerir que los EFs de estas especies no secretan los factores necesarios para la auto-renovación del epiblasto, o tal vez que la etapa gestacional en la que se tomó el tejido para obtener tales "feeder layers" no era equivalente a la etapa funcional en la que se obtienen los EF de ratón (mitad de la gestación). Entre las "feeder layers" heterólogas que se han utilizado con éxito para derivar ESCs se incluyen líneas celulares de hígado de rata Bufalo para ratón (Smith and Hooper 1987); cordón umbilical bovino para caballo (Saito, Ugai et al. 2002); fibroblastos de pulmón fetal humano para vaca (Gjorret and Maddox-Hyttel 2005); y fibroblastos de bazo de trucha arco iris para pez cebra (Fan, Crodian et al. 2004). Pero en la mayoría de los casos en que se requieren "feeder layers", los EF de ratón o bien STO (una línea de fibroblastos de ratón transformada) permiten la obtención de la mayoría de las ESCs de mamíferos y todavía se usan en la actualidad.

Las ESCs murinas crecen como colonias compactas y redondas de células de pequeño tamaño que dependen de la ruta de LIF/STAT3. Tradicionalmente las condiciones de cultivo consistían en una monocapa de EF inactivados que hacía de soporte para las ESCs, y medio de cultivo suplementado con FCS y "Leukemia inhibitory factor" (LIF) (Evans and Kaufman 1981). LIF es una citoquina producida por el endometrio que permite la implantación del blastocisto (Pera and Tam 2010). En ESCs, LIF se une a su receptor, Gp130, y mantiene la auto-renovación y la pluripotencia al fosforilar STAT3 (Williams, Hilton et al. 1988, Niwa, Burdon et al. 1998). El efecto de LIF en el desarrollo *in vitro* de los embriones ha sido ampliamente estudiado, pero los resultados a veces son contradictorios. Algunos trabajos sostienen que LIF incrementa la formación de

blastocistos y reduce la fragmentación embrionaria en el ratón (Tsai, Chang et al. 1999, Cheng, Huang et al. 2004), mientras que otros muestran resultados opuestos, como que al suplementar con LIF el medio de cultivo no mejora el desarrollo embrionario en humano y bovino (Jurisicova, Ben-Chetrit et al. 1995, Gutierrez-Adan, Perez-Crespo et al. 2006). Sin embargo, estos resultados contradictorios pueden deberse a que los experimentos utilizaron diferentes especies y sistemas de cultivo. No obstante, LIF parece tener protagonismo tanto en el mantenimiento de las ESCs como en la diapausa embrionaria. Esta dependencia común de LIF ha constituido un argumento a favor de la teoría de que las ESCs naïve podrían considerarse como un estado similar a la diapausa. Además, la entrada en diapausa incrementa la eficiencia de obtención de ESCs a partir de blastocistos (Brook and Gardner 1997, Welling and Geijsen 2013). Por lo tanto, sería interesante investigar si los embriones cultivados *in vitro* en medio suplementado con LIF son más aptos para la obtención de ESCs.

Posteriormente surgió un innovador sistema de cultivo para la obtención de ESCs en el cual la señalización de la proteína quinasa activada por mitógenos (MAPK/MEK) era eliminada, y la actividad de la quinasa sintetizadora de glucógeno (GSK3) era reducida (Ying, Wray et al. 2008). Este sistema de cultivo combina el uso de LIF con dos inhibidores de quinasas (2i): PD0325901, un inhibidor de las enzimas proteína quinasas activadas por mitógenos MEK1 y MEK2, y CHIR99021, un inhibidor de GSK3β, en un medio de cultivo definido que evita el uso de FCS. El sistema LIF-2i permite la eficiente obtención y expansión de ESCs capaces de transmitir a linaje germinal en diferentes cepas de ratón de las que previamente había sido imposible obtener ESCs (Ying, Wray et al. 2008, Nichols, Jones et al. 2009), y por primera vez, de la rata (Buehr, Meek et al. 2008, Li, Tong et al. 2008, Ying, Wray et al. 2008, Leitch, Blair et al. 2010). Igualmente, al suplementar el medio de cultivo embrionario con 2i, se suprime la formación del hipoblasto, lo que supone que toda la ICM se convierte en epiblasto pluripotente, incrementando la eficiencia de obtención de ESCs (Nichols, Silva et al. 2009). Además, la eficiencia de reprogramación de células somáticas a iPSCs en el ratón se incrementa también al suplementar el medio de cultivo con 2i (Silva, Barrandon et al. 2008).

Por otra parte, se han usado diversos factores de crecimiento para suplementar el medio de cultivo y aislar células pluripotentes que son notablemente parecidas a las ESCs: las EGCs, aisladas a partir de PGCs suplementando el medio de cultivo con LIF, factor de células troncales (“stem cell factor”, SCF) y factor de crecimiento de fibroblastos básico (“basic fibroblast growth factor”, bFGF) (Resnick, Bixler et al. 1992). De la misma manera se han aislado GSCs tras el cultivo *in vitro* de gonocitos o células troncales espermatogénicas (“spermatogonial stem cells”, SSCs) en presencia de LIF, bFGF, factor de crecimiento epidérmico (“epidermal growth factor”, EGF) y factor neurotrópico derivado de células gliales (“glial cell line-derived neurotrophic factor”, GDNF) (Kanatsu-

Shinohara, Ogonuki et al. 2003, Kanatsu-Shinohara, Inoue et al. 2004). Todos estos factores también afectan a la proliferación y mantenimiento de otras poblaciones de células pluripotentes: EGF es un potente mitógeno que incrementa la regeneración tisular en diferentes órganos como la piel, el hígado y el epitelio intestinal en la edad adulta (Reynolds and Weiss 1992); y se ha observado que GDNF estimula la auto-renovación de las células troncales espermatogénicas *in vivo* (Meng, Lindahl et al. 2000).

Curiosamente, estudios recientes han descrito que el transcriptoma de las células pluripotentes depende más de las condiciones de cultivo que de su origen o procedimiento de obtención. Cuando se comparó la expresión génica de células cultivadas en medio con FCS *vs.* cultivadas en el sistema 2i obtenidas en las mismas condiciones se observaron grandes diferencias, pero no al comparar ESCs obtenidas a partir de blastocistos con EGCs obtenidas a partir de PGCs, ambas cultivadas en las mismas condiciones (Marks, Kalkan et al. 2012, Leitch, McEwen et al. 2013).

Por lo tanto, sería de gran interés analizar el proceso de obtención de ESCs bajo diferentes condiciones de cultivo; y en particular bajo condiciones optimizadas para el cultivo de células germinales para así poder probar la teoría de que durante el proceso de obtención de ESCs existe un intermediario similar a una célula germinal.

2. Influencia de las condiciones de cultivo en la estabilidad epigenética y el mantenimiento de la pluripotencia

Una vez establecidas, las líneas de ESCs pueden ser mantenidas en cultivo durante periodos indefinidos; no obstante, en ocasiones esto hace que pierdan su pluripotencia (Nagy, Gocza et al. 1990) debido a la aparición de alteraciones epigenéticas como modificaciones en la metilación de los genes improntados (Fujimoto, Mitalipov et al. 2005, Rugg-Gunn, Ferguson-Smith et al. 2005, Horii, Yanagisawa et al. 2010). En ESCs humanas se ha descrito que el cultivo prolongado afecta a la metilación de la región promotora de genes oncosupresores (Maitra, Arking et al. 2005). Otros estudios indican que el ADN retroviral que se introduce en las ESCs y que es previamente silenciado, puede volver a ser reactivado por una pérdida de metilación en función de las condiciones de cultivo (Allegrucci, Wu et al. 2007, Minoguchi and Iba 2008). Estos cambios son impredecibles y variables entre líneas celulares, y una vez establecidos pueden ser estables y transmitidos a las células diferenciadas a partir de ellas (Lund, Narva et al. 2012). Sin embargo, hasta ahora no se han descrito cuáles serían las condiciones de cultivo óptimas para una mayor estabilidad epigenética.

En el epiblasto temprano del blastocisto, ambos cromosomas X se encuentran aún activos en las hembras, por lo que cabría esperar la misma situación para las ESCs obtenidas a

partir de estos embriones. Sin embargo, se ha encontrado gran variabilidad entre líneas celulares, sobre todo en las ESCs humanas (Bermejo-Alvarez, Ramos-Ibeas et al. 2012). Las condiciones de cultivo también pueden afectar a la inactivación del cromosoma X, ya que cambios en la concentración de oxígeno pueden promover su inactivación (Lengner, Gimelbrant et al. 2010), así como otros estímulos estresantes como la congelación y la descongelación (Lund, Narva et al. 2012).

Por otra parte, algunas líneas XX muestran una pérdida global de metilación del ADN, posiblemente asociada con el hecho de que sus dos cromosomas X se encuentran activos. Todo esto hace que las líneas XX sean más inestables en cultivo que las XY, por lo que en investigación son preferibles las líneas de ESCs XY (Zvetkova, Apedaile et al. 2005, Pannetier and Feil 2007).

Pluripotencia en tejidos adultos

En los últimos años se ha descrito la presencia de células troncales adultas ("adult stem cells", ASCs) que dan soporte y mantienen a múltiples tejidos. Estas células son multipotentes, lo que significa que están limitadas a diferenciarse hacia linajes celulares específicos. Se han identificado ASCs en varios tejidos y órganos: músculo esquelético, hueso, cartílago, piel, vasos sanguíneos, corazón, hígado, intestino, pulmón, tejido graso, pulpa dentaria, fluido amniótico, sangre periférica, epitelio ovárico, testículo y médula ósea (Macaluso and Myburgh 2012). Las ASCs se encuentran en áreas específicas dentro de estos órganos llamadas "nichos"; microambientes especializados donde están protegidas mientras mantienen un bajo índice de división. Estos nichos están compuestos por una matriz extracelular específica y células de soporte, y tienen la capacidad de modular la señalización para promover la auto-renovación o la diferenciación celular (Greco and Guo 2010).

A lo largo de la vida, cierto número de ASCs serán activadas con el fin de mantener la homeostasis del tejido, mientras que otras ASCs permanecerán en un estado quiescente, todas coexistiendo en la misma zona (Li and Clevers 2010). Su localización y su bajo índice de proliferación hace que estas células sean mucho más difíciles de estudiar que otras células pluripotentes como las ESCs (Alvarez, Garcia-Lavandeira et al. 2012). A pesar de ello, hoy en día se han descrito numerosos subtipos de ASCs: células troncales hematopoyéticas y mesenquimales localizadas en la médula ósea, células troncales intestinales en las criptas de Lieberhahn, células troncales del hígado, células troncales del hueso y del cartílago, células troncales del pelo y de la piel, células troncales neuronales, células troncales pancreáticas, células troncales de la retina, células troncales cardiacas, células troncales de la pulpa dentaria y células troncales del músculo

esquelético (Schabort, Myburgh et al. 2009). Todas estas células tienen un gran potencial regenerativo, por lo que se han utilizado en modelos animales de daño tisular. Del mismo modo se ha investigado su habilidad para trans-diferenciarse *in vitro* con el objetivo de utilizarlas para trasplantes (Macaluso and Myburgh 2012). Tras ser purificadas, las ASCs pueden ser cultivadas *in vitro* si se suplementa el medio con factores de crecimiento, micronutrientes, antioxidantes y hormonas a concentraciones fisiológicas (Alvarez, Garcia-Lavandeira et al. 2012).

Probablemente, uno de los subtipos de ASCs más estudiado sean las SSCs. Estas células son capaces de dividirse para producir nuevas SSCs, o de diferenciarse hacia espermatogonias para mantener la espermatogénesis (Kanatsu-Shinohara, Inoue et al. 2011). Aunque en el testículo del ratón las SSCs suponen sólo un 0,03% del total de células (de Rooij and Mizrak 2008), se han desarrollado técnicas que permiten su aislamiento y cultivo a largo plazo dando lugar a las GSCs. Además, se ha descrito la aparición de colonias similares a las ESCs que han sido llamadas células troncales de linaje germinal multipotentes ("multipotent germline stem cells" mGSCs) (Kanatsu-Shinohara, Ogonuki et al. 2003, Kanatsu-Shinohara, Inoue et al. 2004, Guan, Nayernia et al. 2006).

A pesar de que los marcadores característicos de las ESCs como *Oct4*, *Sox2*, *Nanog*, *Klf4*, *Lin28* o *Fut4 (Ssea1)* se expresan en algunos tipos de ASCs como las HSCs (Wang, Zhou et al. 2010), las células troncales mesenquimales ("mesenchymal stem cells", MSCs) (Jaramillo-Ferrada, Wolvetang et al. 2012) y las SSCs (Izadyar, Pau et al. 2008); las ASCs también expresan marcadores específicos en función de la localización del nicho al que pertenecen. Sin embargo, aún se desconocen buenos marcadores comunes a todas las ASCs, o específicos de cada órgano, ya que algunos de ellos se expresan también en células diferenciadas.

Marcadores comunes de pluripotencia y de linaje germinal

1. Especificación y desarrollo del linaje germinal

La especificación del linaje germinal es uno de los primeros eventos de diferenciación celular que se produce en el embrión en mamíferos. La inducción de las PGCs comienza antes de la gastrulación en el epiblasto proximal a día 6,25 de desarrollo embrionario gracias a la estimulación de las proteínas morfogenéticas del hueso ("bone morphogenetic proteins", BMPs); principalmente *Bmp4* y en menor medida *Bmp8b*, que son liberadas por el ectodermo extraembrionario (Lawson, Dunn et al. 1999, Ying, Qi et al. 2001). Estos factores activan el receptor serina-treonina quinasa ACVR1 (*Alk2*) (de Sousa Lopes, Roelen et al. 2004), que induce la fosforilación y la translocación nuclear de los factores de transcripción SMAD1, SMAD5 y SMAD8, permitiendo modular la expresión de ciertos genes (de Sousa Lopes, Hayashi et al. 2007). Estos eventos hacen que un grupo de 4-8 células sobreexprese *Blimp1* y *Prdm14*, los dos determinantes cruciales de la especificación hacia el linaje germinal (Ohinata, Payer et al. 2005, Saitou and Yamaji 2012). Estas células proliferan hasta formar un grupo de 20 que sucesivamente expresa otros marcadores de PGCs como *Fragilis* (*Ifitm3*) y *Stella* (*Dppa3*) a día 7,5. Los factores asociados a la pluripotencia *Pou5f1 (Oct4)* y *Nanog* se encuentran inicialmente en la ICM, pero su expresión se restringe al linaje germinal a partir del día 7,75-8,5 (Young, Dias et al. 2010). Posteriormente, las PGCs comienzan su migración y la colonización de los tubérculos germinales, y su número incrementa hasta 4000 a día 12,5 (Saitou, Barton et al. 2002, Sato, Kimura et al. 2002). Una vez que las PGCs alcanzan las gónadas, otros marcadores específicos del linaje germinal comienzan a expresarse, incluyendo *Ddx4* (Toyooka, Tsunekawa et al. 2000) y *Dazl* (Lin and Page 2005).

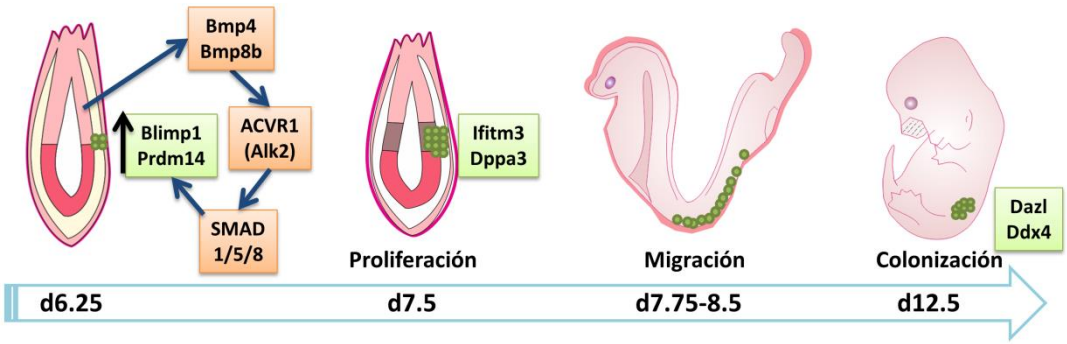

Figura 4. Especificación y desarrollo de las PGCs

2. Marcadores de linaje germinal expresados en células pluripotentes

Existen diferentes tipos de células pluripotentes en función de la fuente celular empleada para su establecimiento: ESCs obtenidas a partir de epiblastos tempranos, EGCs a partir de PGCs, ECCs aisladas a partir de tumores de células germinales, GSCs a partir de células troncales espermatogénicas, e iPSCs a partir de la reprogramación de células somáticas (Xu, Pantakani et al. 2011). Muchas de estas células tienen un origen germinal, excepto las ESCs y las iPSCs, pero todas ellas comparten marcadores de células germinales o premeióticos, lo que podría indicar un origen germinal común (Zwaka and Thomson 2005).

Curiosamente, los principales genes de pluripotencia *Oct4, Sox2* y *Nanog*, parecen ser necesarios para la supervivencia de las células germinales (Kehler, Tolkunova et al. 2004, Yamaguchi, Kimura et al. 2005, Chambers, Silva et al. 2007, Zhao, Ji et al. 2012). Además, varios estudios han demostrado la expresión de marcadores de linaje germinal o premeióticos como *Blimp1*, *Dppa3*, *Ifitm3*, *Piwil2*, *Dazl*, *Ddx4*, *Stra8*, *Rnf17* y *Rnh2* en las ESCs (Geijsen, Horoschak et al. 2004, Qing, Shi et al. 2007, Mise, Fuchikami et al. 2008, Xu, Pantakani et al. 2011). Asimismo un estudio reciente ha asociado *Dazl* con la desmetilación en ESCs mediada por proteínas Tet (ten-eleven translocation) (Blaschke, Ebata et al. 2013). También se ha descubierto otro marcador común de linaje germinal y de pluripotencia, *Prdm14*, cuyo rol principal está relacionado con el establecimiento del linaje germinal (Yamaji, Seki et al. 2008), y que además juega un papel fundamental en la regulación epigenética de la pluripotencia naïve en las ESCs (Yamaji, Ueda et al. 2013).

De la misma manera, estos marcadores de linaje germinal, premeióticos e incluso los marcadores meióticos *Sycp3*, *Pgk2*, y *Creb3/4*, han sido detectados en iPSC, GSCs y EGCs, tanto en líneas celulares masculinas como en las femeninas. Al analizar el proceso de reprogramación de células somáticas, se ha observado que los marcadores germinales *Blimp1, Dppa3* e *Ifitm3* comienzan a expresarse entre los días 6 y 9 de reprogramación, y que su expresión va incrementando hasta el día 22, cuando alcanzan los niveles observados en ESCs. Por el contrario, la expresión de los marcadores de pluripotencia *Pou5f1* y *Sox2* comienza más tarde, a día 12. Otros marcadores premeióticos como *Stra8*, *Dazl* y *Ddx4* se detectan sólo a partir del día 22 de reprogramación (Xu, Pantakani et al. 2011). Además, se ha descrito recientemente una nueva isoforma de splicing alternativo de *Dazl* en el ratón que carece del exón 8, conocida como *Dazl_Δ8*, que se expresa conjuntamente con la isoforma completa de *Dazl* en diferentes tipos de células pluripotentes: ESCs, EGCs, iPSCs, GSCs y ECCs (Xu, Tan et al. 2013).

En embriones preimplantacionales de ratón se ha detectado la expresión de los marcadores de linaje germinal *Dppa3*, *Dazl* y *Ddx4* a través de todos los estadios de

desarrollo (Pan, Liao et al. 2008, Xu, Pantakani et al. 2011). Además, la expresión de *zDazl* se ha localizado en embriones tempranos de pez cebra (Maegawa, Yasuda et al. 1999, Hashimoto, Maegawa et al. 2004), y *xDazl* en embriones de Xenopus hasta el estadio 10 (Houston and King 2000).

Por otra parte, en ASCs se ha descrito la expresión de *DAZL*, *c-Kit* y *POU5F1* en células troncales de fluido amniótico en humanos (Stefanidis, Loutradis et al. 2008). Además, se han localizado *Pou5f1*, *Dppa3*, *Ifitm3*, *Dazl* y *Ddx4* en médula ósea en humanos (Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005). Otro estudio en ratón describió la expresión de *Dazl* en MSCs de la médula ósea, pero a niveles más bajos que al analizar el total de células de la médula ósea (Katsara, Mahaira et al. 2011). Durante el desarrollo postnatal, el sistema hematopoyético está sustentado por las células troncales que se encuentran en la médula ósea (Morrison, Uchida et al. 1995), pero las HSCs se originan en la misma región que las PGCs; el epiblasto proximal. Después, las HSCs colonizan la región de la aorta-gónada-mesonefro para después migrar al hígado fetal (Medvinsky and Dzierzak 1996), al mismo tiempo que las PGCs atraviesan la misma región para colonizar las gónadas fetales (Molyneaux and Wylie 2004).

Tabla 1. Marcadores comunes de pluripotencia y de linaje germinal.

Marcador molecular	Expresión	Referencias
Fut4 (Ssea1)	ESC, PGC, EGC, ECC, iPSC, embrión	(Knowles, Aden et al. 1978, Solter and Knowles 1978, Fox, Damjanov et al. 1981, Zhao, Ji et al. 2012)
c-Kit	ESC, PGC, ECC, HSC	(Zhao, Ji et al. 2012)
UTF1	ESC, ECC, ICM, células germinales	(Kooistra, Thummer et al. 2009, Zhao, Ji et al. 2012)
Pou5f1	ESC, PGC, EGC, ECC, iPSC, ICM, medulla ósea	(Pesce and Scholer 2000, Pesce and Scholer 2001, Kehler, Tolkunova et al. 2004, Zhao, Ji et al. 2012)
Dppa3	ESC, PGC, ECC, iPSC, embrión, médula ósea	(Bowles, Teasdale et al. 2003, Benson, Karsch-Mizrachi et al. 2004, Bortvin, Goodheart et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Xu, Pantakani et al. 2011, Zhao, Ji et al. 2012)
Sox2	ESC, PGC, EGC, ECC, iPSC, embrión	(Botquin, Hess et al. 1998, Boyer, Lee et al. 2005, Zhao, Ji et al. 2012)
GCNF	ESC, ECC, células germinales	(Lei, Hirose et al. 1997, Lan, Xu et al. 2009, Zhao, Ji et al. 2012)

Nanog	ESC, PGC, EGC, ECC, iPSC	(Chambers, Silva et al. 2007, Yamaguchi, Kurimoto et al. 2009)
Prdm14	ESC, PGC	(Yamaji, Seki et al. 2008, Leitch, McEwen et al. 2013, Yamaji, Ueda et al. 2013)
Blimp1	ESC, PGC, iPSC	(Xu, Pantakani et al. 2011)
Ifitm3	ESC, PGC, iPSC, medulla ósea	(Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Xu, Pantakani et al. 2011)
Piwil2	ESC, PGC	(Xu, Pantakani et al. 2011)
Dazl	ESC, PGC, EGC, GSC, ECC, iPSC, embrión, médula ósea	(Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Pan, Liao et al. 2008, Katsara, Mahaira et al. 2011, Xu, Pantakani et al. 2011, Xu, Tan et al. 2013)
Ddx4	ESC, PGC, iPSC, embrión, médula ósea	(Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Xu, Pantakani et al. 2011)
Stra8	ESC, PGC, iPSC	(Xu, Pantakani et al. 2011)
Rnf17	ESC, PGC	(Xu, Pantakani et al. 2011)
Rnh2	ESC, PGC	(Xu, Pantakani et al. 2011)

ESC: células troncales embrionarias; PGC: células primordiales germinales; EGC: células germinales embrionarias; ECC: células embrionarias de carcinoma; iPSC: células de pluripotencia inducida; EGC: células troncales de linaje germinal; CTH: células troncales hematopoyéticas; ICM: masa celular interna

En conclusión, se ha observado que numerosos marcadores de linaje germinal y premeióticos se expresan en varios tipos de células pluripotentes, lo que podría indicar un origen germinal común para todos ellos. Según esta teoría, el blastocisto (d3,5) expresa los principales marcadores de pluripotencia *Pou5f1* y *Sox2*. A lo largo del desarrollo embrionario, la especificación de las PGCs comienza cuando se activa la expresión de *Blimp1*, reprimiendo el programa somático y reactivando las vías de pluripotencia antes de que comience la migración de las PGCs (Saitou 2009). De la misma manera, la obtención de ESCs comienza con el cultivo de blastocistos a día 3,5, cuyas células progresarían hacia el programa de especificación y desarrollo de las PGCs gracias a la señalización de las BMPs, y comenzarían a expresar *Blimp1*, *Ifitm3* y *Dppa3* entre otros marcadores, y a reactivar los genes de pluripotencia para adquirir propiedades de auto-renovación. Del mismo modo, durante la reprogramación de células somáticas mediante los factores *Pou5f1*, *Sox2*, *c-Myc* y *Klf4,* la señalización de las BMPs dispararía la expresión de los marcadores de linaje germinal y finalmente se reactivarían las vías de la pluripotencia (Xu, Pantakani et al. 2011) (Figura 5).

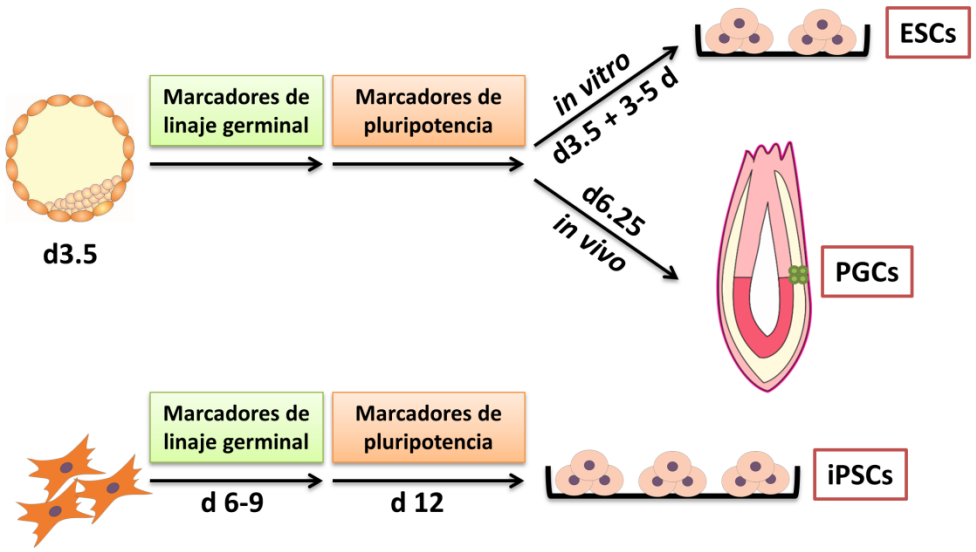

Figura 5. Teoría del origen germinal de las células pluripotentes

La creación de un ratón transgénico en el que la expresión de un gen determinado venga marcada por una proteína flurescente como "Enhanced Green fluorescent protein" (eGFP) constituye un modelo ideal para estudiar la localización de dicho gen en diferentes tejidos o poblaciones celulares en distintos momentos del desarrollo sin interferir con ningún proceso biológico. *Dazl* es uno de los genes cuya principal función está relacionada con el desarrollo del linaje germinal y la meiosis (Ruggiu, Speed et al. 1997), aunque en los últimos años se han publicado estudios que lo relacionan con la pluripotencia (Haston, Tung et al. 2009, Xu, Tan et al. 2013).

Especificación del trofoectodermo

La primera diferenciación de linajes celulares tiene lugar en el blastocisto, cuando las células se diferencian hacia masa celular interna (ICM), precursora del embrión, y trofoectodermo (TE), que dará lugar a la placenta (Schiffmacher and Keefer 2013). Dicha diferenciación está controlada por determinados genes reguladores, pero existen diferencias interespecíficas.

1. Diferencias interespecíficas

En el ratón existe una interacción antagonista entre los genes reguladores de la ICM y del TE; ya que la ICM y su estado pluripotente están mantenidos por *Pou5f1*, mientras que la especificación del TE depende de *Cdx2*, que es capaz de suprimir la expresión de *Pou5f1* y viceversa. Sin embargo, esta situación es muy diferente en los embriones de otras especies de mamíferos. La expresión de *Pou5f1* está restringida a la ICM en el ratón, pero en embriones bovinos y porcinos *Pou5f1* se expresa en el TE también, por lo que el embrión del ratón no es un buen modelo para estudiar la embriogénesis de otras especies como los ungulados (Wolf, Serup et al. 2011). Algunos autores han sugerido que el rápido silenciamiento de *Pou5f1* en roedores podría haber evolucionado para permitir una veloz diferenciación del TE y un periodo preimplantacional más corto, mientras que en otros mamíferos en los que el desarrollo preimplantacional es más largo no habría esta necesidad (Berg, Smith et al. 2011).

Los precursores de las células diferenciadas de la placenta son las células troncales del trofoblasto (“trophoblast stem cells”, TSCs). Su existencia se supone en todos los mamíferos euterios, especialmente durante los estadios tempranos del desarrollo de la placenta cuando el crecimiento del TE es máximo. Sin embargo, se sabe poco acerca de su localización y fenotipo en otras especies diferentes al ratón y al macaco Rhesus (Roberts and Fisher 2011). En el ratón, el nicho de TSCs parece estar localizado en el ectodermo extraembrionario adyacente al epiblasto, del que depende para nutrirse de factores de crecimiento esenciales. Este nicho tan especializado es transitorio y existe sólo durante 3 o 4 días durante el desarrollo postimplantacional. Las TSCs de ratón han sido aisladas a partir del cultivo *in vitro* de explantes de TE polar, obtenidos a partir una región adyacente al epiblasto, sobre una monocapa de fibroblastos mitóticamente inactivados y con medio suplementado con FCS, factor de crecimiento fibrobástico 4 (“fibroblast growth factor”, FGF4) y heparina (Tanaka, Kunath et al. 1998). Del mismo modo se han obtenido TSCs a partir de blastocistos de macaco Rhesus (Vandevoort, Thirkill et al. 2007).

En algunas especies domésticas como el cerdo, la oveja y la vaca, se produce una enorme elongación del TE antes de la implantación, con el objetivo de aprovechar al máximo las

secreciones uterinas a lo largo de un gran área en el lumen uterino (Bindon 1971, Geisert, Brookbank et al. 1982, Thatcher, Meyer et al. 1995). A diferencia del ratón, el TE polar que recubre el disco embrionario en estas especies desaparece rápidamente y no contribuye a la formación de la placenta, mientras que el TE mural es el responsable de la elongación. Se desconoce si la elongación del TE mural depende de una población pluripotente que se encontraría alrededor del disco embrionario, o de células pluripotentes diseminadas a lo largo de todo el TE; o si por el contrario este gran crecimiento se debe a la proliferación de células progenitoras que se encuentran parcialmente diferenciadas (Roberts and Fisher 2011). En consecuencia, no han podido aislarse TSCs de ninguna especie de ungulados; sin embargo se han conseguido aislar líneas celulares de trofoectodermo en oveja y cabra (Miyazaki, Imai et al. 2002), cerdo (Ramsoondar, Christopherson et al. 1993, Flechon, Laurie et al. 1995, La Bonnardiere, Flechon et al. 2002) y vaca (Talbot, Caperna et al. 2000, Shimada, Nakano et al. 2001, Suzuki, Koshi et al. 2011).

2. Aislamiento de líneas celulares de trofoblasto bovino

Las células del trofoblasto crecen *in vitro* formando una monocapa en la que a veces aparecen estructuras con forma de caverna que acumulan líquido en su interior hasta transformarse en esferoides que son liberados al medio de cultivo.

Las primeras líneas de trofoblasto bovino fueron producidas por Talbot *et al.*, a partir de blastocistos de día 7-8 producidos *in vitro* y cultivados sobre una monocapa de fibroblastos STO (Talbot, Caperna et al. 2000). Sin embargo, el co-cultivo con fibroblastos murinos tiene el riesgo de contaminar las células de trofoblasto bovino, y esto podría ser perjudicial para el uso de estas líneas celulares en estudios posteriores. Más tarde, Shimada *et al*. (Shimada, Nakano et al. 2001) establecieron una línea de células de trofoblasto bovino en ausencia de fibroblastos, utilizando medio condicionado por fibroblastos endometriales, pero su eficiencia de obtención fue muy baja; plaquearon más de 50 blastocistos obteniendo sólo una línea celular. El mayor problema parecía radicar en la adherencia del blastocisto a la placa de cultivo y en la proliferación inicial. Desde entonces, la mayoría de los estudios de señalización y diferenciación del trofoectodermo se han llevado a cabo con estas dos únicas líneas celulares producidas por Talbot y Shimada (Nakano, Shimada et al. 2002, Hirata, Sato et al. 2003, Ushizawa, Takahashi et al. 2005, Michael, Wagner et al. 2006, Das, Ezashi et al. 2008, Nakaya, Kizaki et al. 2009, Bai, Sakurai et al. 2011, Schiffmacher and Keefer 2012, Schiffmacher and Keefer 2013). Por lo tanto, sería muy útil conseguir un número mayor de líneas celulares de trofoblasto apropiadas para estudios *in vitro*, que carezcan de posibles contaminaciones por otros tipos celulares.

Se han obtenido líneas celulares de trofoblasto a partir de embriones producidos mediante SCNT o por partenogénesis, y se han observado diferencias significativas en cuanto a su eficiencia de obtención, ya que los embriones partenogenéticos mostraban un menor índice de formación de colonias que los embriones producidos por IVF o por SCNT. De la misma manera, la cantidad de interferón tau (IFN-t), proteína necesaria para el establecimiento de la gestación, producida por las líneas de células del trofoblasto procedentes de embriones partenogenéticos y de SCNT, era menor que la de aquellos producidos por IVF. Además, las líneas procedentes de SCNT contaban con menor cantidad de proteínas anexinas, relacionadas con el mantenimiento de la placentación (Talbot, Powell et al. 2007, Talbot, Powell et al. 2008, Talbot, Powell et al. 2010). Por lo tanto, las líneas celulares trofoblásticas podrían reflejar las posibles alteraciones embrionarias y ser utilizadas como un modelo para su estudio.

En un estudio más reciente, basándose en que el aporte exógeno de BMP4 estimula a las ESCs para convertirse en células trofoblásticas (Murohashi, Nakamura et al. 2010), Suzuki *et al*. utilizaron BMP4 para producir líneas de células trofoblásticas de bovino, pero no encontraron diferencias significativas en la eficiencia de obtención, y sólo pudieron obtener 12 líneas celulares a partir de 172 embriones. Al analizar la expresión de los genes necesarios para el establecimiento de la gestación: interferón tau (*IFNt*), lactógeno placentario (*CSH1*), proteína relacionada con la prolactina 1 (*PRP1*) y glicoproteína asociada a la gestación (*PAG1*), encontraron patrones de expresión muy variables entre las diferentes líneas celulares (Suzuki, Koshi et al. 2011). Por lo tanto, existe una alta variabilidad entre las líneas de células trofoblásticas obtenidas bajo condiciones idénticas.

Muchas de las líneas de células trofoblasticas obtenidas en diferentes estudios crecen continuamente sin mostrar signos de envejecimiento y expresan *POU5F1* (Suzuki, Koshi et al. 2011, Schiffmacher and Keefer 2013), por lo que es posible que estas células representen la población celular pluripotente a partir de la cual se diferencia la placenta en el bovino, o quizás un equivalente a las TSCs de ratón algo más diferenciado.

Las líneas de células trofoblásticas constituyen un modelo muy útil para los estudios de implantación y placentogénesis, y podrían reflejar las características genéticas y epigenéticas y las alteraciones presentes en los embriones de los que proceden. Sin embargo, tan sólo un reducido número de líneas celulares se utiliza para investigación debido a la dificultad de establecer nuevas líneas. Por ello, sería interesante establecer un sistema para la obtención de líneas de células trofoblásticas de manera eficaz a partir de una biopsia de TE que permitiera al embrión seguir siendo viable, mientras sus características o alteraciones son analizadas en su correspondiente línea de células trofoblásticas.

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Objectives

General objective

Although over the last few decades much research has been done on pluripotent cells in mammals due to their numerous applications, most critical aspects remain unknowk, such as the optimal conditions for their identification, isolation and *in vitro* culture. Thus, in this thesis, several determinant aspects for cell lines derivation, as the effect of embryonic source or culture conditions, have been analyzed. Furthermore, the theory that all pluripotent cells may share a common germinal origin has been corroborated, and the ability of the germ cell-specific marker *Dazl* to identify pluripotent cells in adult tissues has been analyzed. Finally, an efficient system to derive bovine trophoblastic cell lines from embryo biopsies has been developed in order to perform trophectoderm and placentation studies, and for genomic and epigenetic diagnosis before embryo transfer.

Specific objectives

1. To analyze if ESCs derivation is affected by embryonic source, by examining ESCs derivation efficiency and by analyzing the characteristics of ESCs derived from bad quality blastocysts produced by ICSI with DNA fragmented sperm.

2. To analyze if ESCs lines can be used for embryonic quality testing and as a safety indicator of the methodology used for embryo production.

3. To determine the effect of culture medium on ESCs derivation and to investigate the existence of an intermediate state during the process that shows germline markers expression.

4. To analyze the efficiency of the germline-specific gene *Dazl* to track the existence of pluripotent cells during *in vivo* development, in adult tissues and during *in vitro* differentiation.

5. To develop a system to efficiently derive bovine trophoblastic cell lines from embryo biopsies as a model for trophectoderm and placentation studies, and for embryonic genomic and epigenetic diagnosis.

Objetivos

Objetivo general

A pesar de que durante las últimas décadas se ha producido un enorme avance en el conocimiento sobre las células pluripotentes en mamíferos, aún se desconocen con precisión muchos aspectos esenciales, como cuáles son las condiciones óptimas para su identificación, aislamiento y cultivo *in vitro*. Por ello, en esta tesis se han analizado algunos aspectos determinantes para la obtención de líneas celulares, como son el efecto de la calidad de la fuente embrionaria o de las condiciones de cultivo. Además se ha contrastado la teoría de que las células pluripotentes podrían compartir un origen germinal común, y se ha analizado la utilidad del marcador de células germinales *Dazl* para identificarcélulas pluripotentes en tejidos adultos. Finalmente, se ha examinado si es posible desarrollar una metodología eficiente para obtener líneas celulares de trofoblasto bovino a partir de biopsias embrionarias que pudieran ser utilizadas tanto para estudios de trofectodermo y placentación, como para el diagnóstico genómico y epigenético del embrión antes de su transferencia.

Objetivos específicos

1. Analizar si la obtención de ESCs se ve afectada por la calidad de la fuente embrionaria mediante el análisis de la eficiencia de obtención de ESCs y las características de las ESCs obtenidas a partir de blastocistos de “mala calidad” producidos mediante ICSI con espermatozoides con ADN fragmentado.
2. Analizar si las líneas de ESCs pueden ser utilizadas para el diagnóstico de la calidad embrionaria y como indicador de la seguridad de la metodología utilizada para la producción de los embriones a partir de los cuales se generan las ESCs.
3. Determinar el efecto del medio de cultivo en la obtención de ESCs e investigar si la transición de embrión a ESCs es mediada por un estadio intermedio con expresion de marcadores de células germinales.
4. Analizar la utilidad del marcador de células germinales *Dazl* para identificar células pluripotentes durante el desarrollo *in vivo*, en tejidos adultos y en diferenciación *in vitro*.
5. Desarrollar un sistema para obtener de manera eficiente líneas de células trofoblásticas bovinas a partir de biopsias embrionarias como modelo para estudios de trofectodermo y diagnóstico génomico y epigenético del embrión.

Intracytoplasmic sperm injection using DNA-fragmented sperm in mice negatively affects embryo-derived ES cells, reduces the fertility of male offspring and induces heritable changes in epialleles

Priscila Ramos-Ibeas, Alexandra Calle, Raúl Fernández-González, Ricardo Laguna-Barraza, Eva Pericuesta, Antonia Calero, Miguel Ángel Ramírez and Alfonso Gutiérrez-Adán

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Chapter I / Capítulo I

Summary

The *in vitro* fertilization procedure intracytoplasmic sperm injection (ICSI) is currently one of the most commonly used methods to overcome male infertility. However, concern has been recently raised that ICSI bypasses the natural selection process of the fertilizing sperm, allowing sperm with fragmented DNA to fertilize an oocyte, and generating embryos with genetic and epigenetic abnormalities. Furthermore, the sperm of infertile men frequently shows DNA strand breaks or other types of DNA damage. Therefore, ICSI using DNA-fragmented sperm (DFS-ICSI) in mice was used as a model to generate bad quality-embryos.

This chapter examines whether embryonic stem cells (ESCs) derived from DFS-ICSI embryos reflect the abnormalities that would be observed in the DFS-ICSI progeny. DFS-ICSI embryos showed a lower ESCs derivation efficiency, although ESCs lines had normal karyotype. Gene expression alterations were observed at early passages, though these differences were lost after several passages.

Furthermore, it was observed that DFS-ICSI reduces fertility in the male progeny and affects the postnatal expression of a defined epigenetically sensitive allele, and this modification may be inherited across generations.

In conclusion, ESCs derivation is affected by embryo features, and embryo abnormalities are transmitted to their corresponding ESCs lines. However, ESCs cannot be used as a model because these alterations are amended along passages.

Resumen

El procedimiento de fertilización in vitro conocido como ICSI (inyección intracitoplasmática de espermatozoides) es actualmente uno de los métodos más utilizados para tratar la infertilidad masculina. Sin embargo, recientemente se ha cuestionado la seguridad de la ICSI ya que evita el proceso de selección natural del esperma, permitiendo que cualquier espermatozoide con ADN fragmentado ("DNA fragmented sperm", DFS) pueda fertilizar un ovocito y generar embriones con anormalidades genéticas y epigenéticas. Además, el esperma de los hombres infértiles con frecuencia muestra roturas u otros daños del ADN. Por lo tanto, se ha utilizado un modelo murino de ICSI con espermatozoides con DFS (ICSI-DFS) para generar embriones de "mala calidad".

Este capítulo analiza si las ESCs obtenidas a partir de embriones ICSI-DFS reflejan las anormalidades que se observarían en los animales generados mediante ICSI-DFS. Los embriones DFS-ICSI mostraron una menor eficiencia de obtención de ESCs, aunque las líneas de ESCs generadas exhibieron cariotipos normales. En pases celulares tempranos pudieron observarse alteraciones en la expresión génica que fueron corregidas durante el cultivo a largo plazo.

Por otra parte se observó que la ICSI-DFS reduce la fertilidad en los machos de la descendencia, y que afecta a la expresión postnatal de un alelo definido epigenéticamente cuya modificación puede ser heredada transgeneracionalmente.

En conclusión, la obtención de ESCs se ve afectada por las características del embrión, y las anormalidades de los embriones son transmitidas a sus correspondientes líneas de ESCs. Sin embargo, las ESCs no pueden ser usadas como un modelo porque las alteraciones producidas se corrigen a través de sucesivos pases celulares.

Intracytoplasmic sperm injection using DNA-fragmented sperm in mice negatively affects embryo-derived embryonic stem cells, reduces the fertility of male offspring and induces heritable changes in epialleles

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Abstract

Intracytoplasmic sperm injection (ICSI) in mice using DNA-fragmented sperm (DFS) has been linked to an increased risk of genetic and epigenetic abnormalities both in embryos and offspring. This study examines: whether embryonic stem cells (ESCs) derived from DFS-ICSI embryos reflect the abnormalities observed in the DFS-ICSI progeny; the effect of DFS-ICSI on male fertility; and whether DFS-ICSI induces epigenetic changes that lead to a modified heritable phenotype. DFS-ICSI-produced embryos showed a low potential to generate ESC lines. However, these lines had normal karyotype accompanied by early gene expression alterations, though a normal expression pattern was observed after several passages. The fertility of males in the DFS-ICSI and control groups was compared by mating test. Sperm quantity, vaginal plug and pregnancy rates were significantly lower for the DFS-ICSI-produced males compared to *in vivo*-produced mice, while the number of females showing resorptions was higher. The epigenetic effects of DFS-ICSI were assessed by analyzing the phenotype rendered by the *Axin1*^{Fu} allele, a locus that is highly sensitive to epigenetic perturbations. Oocytes were injected with spermatozoa from *Axin1*$^{Fu/+}$ mice and the DFS-ICSI-generated embryos were transferred to females. A significantly higher proportion of pups expressed the active kinky-tail epiallele in the DFS-ICSI group than the controls. In conclusion: 1) ESCs cannot be used as a model of DFS-ICSI; 2) DFS-ICSI reduces sperm production and fertility in the male progeny; and 3) DFS-ICSI affects the postnatal expression of a defined epigenetically sensitive allele and this modification may be inherited across generations.

Introduction

The *in vitro* fertilization procedure intracytoplasmic sperm injection (ICSI) is currently the most commonly used method to overcome male infertility. However, concern has been recently raised that ICSI bypasses the natural selection process of the fertilizing sperm [1], allowing sperm with fragmented or otherwise damaged DNA to fertilize an oocyte [2]. Sperm DNA integrity is crucial for paternal reproductive potential and many studies have shown that the sperm of infertile men have more DNA strand breaks or other types of DNA damage than the sperm of fertile donors [3,4]. We recently reported that ICSI using DNA-fragmented sperm (DFS) gives rise to genetic and epigenetic alterations in preimplantation embryos. These modifications include delayed male pronucleus demethylation, different sized telomeres, altered gene expression at the blastocyst stage, and modified expression of imprinting genes [5]. However, some of these suboptimal blastocysts are capable of implantation and our data suggest that the use of DFS for ICSI can produce effects later on in life such as aberrant growth, premature ageing, abnormal behavior, and mesenchymal tumors [5]. However, it is not yet known if ICSI may affect the fertility of the adult male offspring.

Embryonic stem cells (ESC) are clonal populations of cultured cells derived from the blastocyst-stage embryo that can give rise to all of the cell types that constitute the adult organism, and offer an *in vitro* model for early development and diseases, thus enabling teratogenicity testing in a cell culture system and enabling the generation of disease-specific cell lines [6]. Human ESC are generally generated form blastocysts produced by *in vitro* manipulations such as IVF and ICSI; however, *in vitro* manipulated embryos may already possess abnormalities that can be maintained in the ESCs lines generated from these embryos. On the other hand, if ESCs reflects the abnormalities of the embryo, they could be used as a method of testing quality of the embryos produced by assisted reproduction techniques. Moreover, these ESCs could enable us to analyze possible causes of the anomalies observed in adults generated by assisted reproductive technologies without the need to generate new animals.

Recently, it has been reported that ICSI procedures produce primary epimutations in mice that are, nevertheless, corrected in the germ line by epigenetic reprogramming and thus not propagated to subsequent generations [7]. However, ICSI using DFS can cause secondary epimutations or affect metastable epialleles and these abnormalities are transmitted to following generations. Many of these epialleles are comprised of transposable elements, and half of each mammalian genome is made up of these mobile, repetitive elements [8]. In the present study, we examined the mouse metastable epiallele *Axin 1* fused (*Axin1^{Fu}*). This epiallele has a well-characterized locus, whose methylation pattern determines dramatic phenotypic outcomes. The *Axin1^{Fu}* allele seems

to be particularly vulnerable to environmental factors and its modifications may persist across several generations [9] through a process known as transgenerational epigenetic inheritance. *Axin1*^{Fu} is a dominant gain-of-function allele that has a 5.1-kb intracisternal-A particle (IAP) retrotransposon (subtype I1) inserted in an antisense direction in intron 6 of *Axin1*[10]. The *Axin 1* gene regulates embryonic axis formation in vertebrates by inhibiting the Wnt signaling pathway [11]. The characteristic *Axin1*^{Fu} phenotype consists of kinks in the tail caused by axial duplications during embryogenesis [11]. This phenotype is variably expressed among *Axin1*^{Fu} individuals, and this variable expressivity correlates with differential DNA methylation at a cryptic promoter within the long terminal repeat (LTR) sequence of the IAP inserted in intron 6 of *Axin1*[12]. Rakyan *et al.* [12] observed that the methylation state of *Axin1*^{Fu} in mature sperm reflects the methylation state of the allele in the somatic tissue of the animal, suggesting that it is not epigenetically reprogrammed during gametogenesis. In prior studies, we detected that ICSI causes epigenetic defects in preimplantation mouse embryos [13,14,15]. However, we are unaware if some of these epigenetic effects of ICSI will persist in subsequent generations when mice produced by ICSI are naturally mated.

This study was designed to determine whether the epigenetically inherited *Axin1*^{Fu} allele is also sensitive to preimplantation development alterations induced by DFS-ICSI by examining whether DFS-ICSI causes a shift in *Axin1*^{Fu} epiallele expression in the resulting progeny that is inherited by the next generation. In addition, we assessed the fertility of male mice generated by DFS-ICSI and determined whether ESC derived from blastocysts generated by DFS-ICSI differed from ESC derived from *in vivo* produced blastocysts as a possible method of testing assisted reproduction techniques without the need to generate new animals.

Materials and methods

Ethics statement

All experimental procedures using mice were approved by our Institutional Review Board (INIA), permit number CEEA2012/021, and performed according to the *Guide for Care and Use of Laboratory Animals* endorsed by the *Society for the Study of Reproduction* and European legislation.

Animals and Embryo Production

Mice were fed a standard diet (Harland Ibérica) *ad libitum* and kept in a temperature- and light controlled room (22-24ºC, 14L:10D). B6D2F1 (C57BL/6 x DBA/2) female mice (8–10 weeks old) were superovulated by intraperitoneal injection of 7.5 IU of equine chorionic gonadotropin (eCG; Foligon 5000 Intervet), followed 48 h later by 7.5 IU of human chorionic gonadotropin (hCG; Veterin Corion, Equinvest) [16]. Oocytes in the superovulated B6D2F1 females were obtained from the ampulla of the oviduct and fertilized by ICSI using the DFS of B6D2F1 males [5] to generate ICSI embryos and then transferred at 2-cell stage to generate animals to study fertility. Embryos produced by B6D2F1 females naturally mated with B6D2F1 males and *in vitro* cultured from zygote to 2-cell, were used as controls. In the experiment to analyze postnatal expression of an epigenetically sensitive allele, oocytes obtained from B6D2F1 superovulated females were fertilized with the sperm of 129/Rr *Axin1*$^{Fu/+}$ males to generate an ICSI *Axin1*^{Fu} group [9]. Animals produced by natural mating of B6D2F1 females with 129/Rr *Axin1*$^{Fu/+}$ males formed the *Axin1*^{Fu} control *in vivo* group. In addition, a 2-cell embryo transfer *Axin1*^{Fu} group was set up using zygotes obtained 0.5 day p.c. from the uterus of superovulated B6D2F1 females naturally mated with 129/Rr *Axin1*$^{Fu/+}$ males. All embryos obtained were cultured in KSOMaa + BSA for 24 h and those reaching the 2-cell stage were transferred to CD1 pseudopregnant females [17]. The tail phenotypes of the ICSI *Axin1*^{Fu} group, 2-cell embryo transfer *Axin1*^{Fu} group, and natural mating offspring group were analyzed and genotyped [9] to establish kinkiness categories. The tail phenotypes of the obtained pups were classified as no visible kink or slight kinking (1) (a small kink forming an angle <45º to the main tail axis), medium kinking (2) (one kink >30º but <45º), and kinky or very kinky (3) (several kinks >45º). The pups were genotyped by multiplex PCR of genomic DNA [12].

ICSI using Frozen-Thawed Sperm

ICSI was performed as previously described [18]. Epididymal sperm cells collected in a minimal volume for freezing-thawing were placed in the bottom of a 1.5-ml polypropylene centrifuge tube and overlaid with the volume of fresh medium necessary

to obtain a final concentration of 2.5 million cells per ml. The sperm extender used did not contain cryoprotectants such as EDTA or EGTA to induce DNA fragmentation [2]. Sperm samples were frozen in liquid nitrogen and stored for periods ranging from 1 day to 4 weeks at -80˚C. Asepsis was maintained throughout the procedure. A volume of frozen-thawed sperm cells was mixed with 5 volumes of a 10% solution of polyvinyl-pyrrolidone (PVP; Mw 360,000) in M2 to give a final volume of 40-50 µl and placed in a culture dish for microinjection. ICSI was performed in M2 medium at room temperature. Sperm were mixed with M2 medium containing 10% PVP to reduce stickiness. Individual sperm heads, either mechanically obtained by decapitation using the piezo unit (for fresh sperm) or by freezing/thawing, were injected into oocytes as groups of ten oocytes. After a 15 min recovery period at room temperature in M2 medium, surviving oocytes were returned to mineral oil-covered KSOM and cultured at 37ºC in a 5% CO2 air atmosphere for up to 24 h. Embryos that reached the 2-cell stage were transferred to the oviduct of Day 0.5 pseudopregnant females.

Male Fertility Tests

Three virgin female B6D2F1 mice of 8-12 weeks of age were partnered with each male produced by ICSI or by natural mating on 5 consecutive days. The males in each group were classified according to age as young (4-6 months), adult (10-12 months) or old (16-18 months age) (control group: N=16, N=10 and N=14; ICSI group: N=16, N=23, and N=14 respectively). Every day during cohabitation, females were examined for plugs as evidence of mating. On gestation Day 14, females were euthanized using CO_2 and the variables percentage of pregnant females, number of vaginal plugs, resorptions per litter and litter size recorded. Live fetuses were euthanized after examination. This fertility study was repeated 2-3 times.

Sperm Motility

Adult 9-month-old ICSI males were sacrificed by cervical dislocation. The testis, epididymis, and vas deferens were immediately removed, and fat and veins dissected away to avoid contamination. For the motility test, the sperm were harvested into a 35 mm-well containing 500 µl of M2 medium (Sigma-Aldrich) by exerting soft pressure from the cauda epididymis to the end of the vas deferens with the help of watchmaker's tweezers. The sperm sample was incubated at 37ºC for 15 min until the sperm were homogeneously distributed in the M2 drop. A sample of 25 µl from the surface of the drop (swim-up) was placed on a microscope slide to obtain quantitative sperm motility variables. Sperm motility and progressive motility measurements were analyzed using an Integrated Semen Analysis System (ISAS). The parameters used for this analysis were SPV (Smoothed Path Velocity), TV (Track Velocity), STR (Straightness: ratio of VSL/VAP) and

ALH (Amplitude of Lateral Head displacement), based on total motility, progressive motility and speed (static, medium and slow sperm cells) [19]. For sperm counts, a sample of sperm was diluted 1/10 in milli-Q water and 10 µl were placed in a Bürker chamber to obtain sperm cells concentrations (million spermatozoa/ml) using a standard procedure.

Histological and TUNEL Assessment of the Testes

Both testes were fixed in Bouin's solution for 24 h. The immersion-fixed testes were processed for paraffin embedding and posterior sectioning. Sections (5-µm thick) across the seminiferous tubules were deparaffinated, hydrated and stained with hematoxylin for histological examination. The TUNEL assay for apoptotic cell detection was performed using the *In Situ* Cell Death Detection Kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. Apoptosis was visualized using anti-fluorescein antibody Fab fragments conjugated with alkaline phosphatase (AP) and converter-AP. The number of TUNEL positive cells in approximately 250 seminiferous tubules of each mouse was counted, and apoptotic indices then determined by calculating the ratio of the total number of TUNEL positive cells/number of counted seminiferous tubules. For histological examination, seminiferous tubule cross sections were randomly chosen in three non-serial sections per animal, totaling more than 100 tubules/animal and the percentage of tubules showing abnormal spermatogenesis (irregularly outlined seminiferous tubules showing disarranged cell layers and loss of germ cells, or premature release of germ cells into the seminiferous tubule lumen, or an empty tubular lumen) and abnormal tubule morphology (empty tubules and seminiferous tubules containing only Sertoli cells) compared in the control (N=5) and ICSI (N=8) groups. Since germ cell numbers vary in tubule sections between stages I-VIII and stages IX-XIV, the same number of tubules at each of these two stages was considered per animal.

Embryonic Stem Cell Production and Karyotyping

Female mice (8–10 weeks old) were superovulated as described above [16], and *in vivo*-produced blastocysts were collected 3.5 days after the hCG injection and used as controls. ICSI-produced blastocysts were obtained as described above. The *in vivo*- and ICSI-produced blastocysts were plated individually onto 96-well plates containing mitomycin-C treated (Sigma-Aldrich corporation St. Louis, MO, USA) mouse embryonic fibroblast (MEF) cells on 0.1% gelatin-coated tissue plates containing Dulbecco's modified Eagle medium (DMEM plus 4500 mg/l glucose, glutaMAX, and pyruvate; Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS (PAA Laboratories Cölbe Germany), 2 mM glutamine, 1 mM MEM nonessential amino acids solution, 1 mM β-mercaptoethanol, 1000 U/ml LIF, an antibiotic mixture containing 100 U/ml penicillin and 100 µg/ml streptomycin, 3 µM GSK3Beta inhibitor (Stemolecule CHIR99021, Stemgent, San Diego,

CA, USA) and 0.5 µM MEK inhibitor (Stemolecule PD0325901, Stemgent, San Diego, CA, USA). Blastocysts were allowed to attach to supportive MEFs and to expand for four days. After this, all cell clumps were disaggregated by incubation in 0.05% Trypsin / 0.02% EDTA in Ca^{2+}-free and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) at 37ºC for 3 min and transferred into 96-well plates containing MEFs and ES medium. Approximately 4 days after trypsinization, compact ESC colonies could be detected and these were then trypsinized into 24- well plates containing MEFs and ES medium lacking GSK3Beta and MEK inhibitors. For cell line expansion, cells were trypsinized at 80% confluence, and clones not reaching confluence plated onto the same plate size. When ESCs were transferred to a 35-mm dish, this was considered the first passage. The culture medium was changed daily.

For karyotyping, ESC were arrested in metaphase by supplementing the culture medium with 0.1 µg/ml Karyomax Colcemid Solution (Gibco, Paisley, Scotland, UK) for 2 h at 37°C in a 5% CO2 air atmosphere. Cells were then disaggregated by incubation in 0.05% Trypsin / 0.02% EDTA in Ca^{2+}-free and Mg^{2+}-free PBS at 37ºC for 2 min. After pipetting, a single cell suspension was washed twice in PBS by centrifugation at 200 G for 5 min. The pellet obtained was subjected to hypotonic shock by resuspending in 0.075 M KCl for 15 min at 37°C. After a second centrifugation step, the hypotonic solution was removed and the pellet was fixed in a methanol/acetic acid solution (3:1; vol/vol) by gently pipetting. Ten minutes later, cells were re-pelleted and fixed for a second time. Before slide mounting, cells were washed twice with PBS. The slides were dried overnight at 55°C, stained in freshly made 10% Giemsa solution for 30 min, and rinsed with distilled water. Finally, chromosome spreads were observed using an Optishot II microscope (Nikon, Tokyo, Japan) at a magnification of 1000x [20].

RNA Isolation, cDNA Synthesis, and qPCR

Poly (A) RNA was extracted from 7 ICSI-derived and 7 *in vivo*-derived ESC lines at passage 0 and passage 10 using the Dynabeads mRNA Purification Kit (Life Technologies, Oslo, Norway) following the manufacturer's instructions with minor modifications. Briefly, 100 µl of lysis buffer were added to the sample and incubated at RT for 10 min with gently shaking. Then, 20 µl of beads were added and incubated at RT for 5 min with gentle shaking, allowing beads/mRNA complexes formation. Finally, beads/mRNA complexes were washed twice in washing buffer A and twice in washing buffer B, and resuspended in 10 mM Tris-HCl pH 7.5. Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure, allowing Random Primer and Oligo dT annealing, and the RT mix was then completed with the addition of 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNAsin RNAse inhibitor (Promega, Madison, WI, USA),

MMLV HP RT 10X reaction buffer, 5 mM DTT and 5 U MMLV high performance reverse transcriptase (Epicentre, Madison, WI, USA). Tubes were first incubated at room temperature for 10 min and then at 42°C for 60 min to allow the reverse transcription of RNA, followed by 70°C for 10 min to denature the RT enzyme. To detect each transcript, we used 2 µl of the cDNA sample in the RT-PCR. mRNA transcripts were quantified by real-time qRT-PCR [21]. Two replicate PCR experiments were conducted for all genes of interest. Experiments were designed to compare the relative levels of each transcript and those of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) in each sample. PCR was performed by adding a 2-µl aliquot of each sample to the PCR mix (GoTaq qPCR Master Mix, Promega, Madison, WI, USA) containing the specific primers. Primer sequences are provided in Table S1.

Table S1. Primers used for RT-PCR

Gene	Primer sequences 5´-> 3´
H2az	AGGACGACTAGCCATGGACGTGTG / CCACCACCAGCAATTGTAGCCTTG
Sox2	GCACATGAACGGCTGGAGCAACG / TGCTGCGAGTAGGACATGCTGTAGG
Kap1	GGAATGGTTGTTCATTGGTG / ACCTTGGCCCATTTATTGATAAAG
Mecp2	ATATTTGATCAATCCCCAGGG / CTTAGGTGGTTTCTGCTCTC
Setdb1	CTTCTGGCTCTGACGGTGAT / GGAAGCCATGTTGGTTGATT
Hdac10	GTGCCTGCTTAGGAGCTCTG / CCTCCACCCTACAGAATTGG
Dnmt1	GCTTCTACTTCCTCGAGGCCTA / GTTGCAGTCCTCTGTGAACACTGTG
Dnmt3a	CACAGAAGCATATCCAGGAGTG / GTCCTCACTTTGCTGAACTTGGC
Dnmt3b	ACGTCAATCCTGCCCGCAAAGGT / ACTGGGTTACATGCCAGGAATCTT
Osgin2	TCCGGCCTTACTGCCGCTGA / TGGCTGGCTTGAGTTACGGCC
Xrcc1	AGAATGGCGAGGACCCGTAT / CTCTGGGATTGGCAGGTCAG
Ercc1	GTGCTGCTGGTTCAAGTGGA / GCAGTCAGCCAAGATGCACAT
Xpa	AATGCGGGAAAGAGTTCATGG / CATCAGCATCTCTGCAGCTGT
Ddit4	CTCTTGTCCGCAATCTTCGCT / GGACACCCCATCCAGGTATGA
Gadd45b	CTTCTGGTCGCACGGGAAGG / GCTCCACCGCGGCAGTCACC
Alkbh3	GTGGACTGGCACAGCGACGA / CCAGTCGGCTTGTGTGGCTCC
Alkbh8	AGGAAGGCCACACCTCCATCCC / CAGCCAGCGCATCGCAGACTA

The comparative cycle threshold (CT) method was used to quantify expression levels [22]. Quantification was normalized to the endogenous control *Gapdh*. Fluorescence was acquired in each cycle to determine the threshold cycle, or the cycle during the log-linear phase of the reaction wherein fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the CT value was determined by subtracting the *Gapdh* CT value for each sample from the CT value of each gene in the sample. CT was calculated using the highest sample CT value (i.e., the sample with the lowest target expression) as an arbitrary constant to be subtracted from all other CT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2^{-CT}.

Statistical Analysis

Pregnancy rates, number of implantations per litter, numbers and percentages of live births, resorptions, and dead fetuses per litter were compared between the different groups by one-way ANOVA and mean comparisons made using Holm-Sidak post hoc tests. The effect of treatment on tail phenotype was analyzed by multinomial logistic regression (SigmaStat, Jandel Scientific, San Rafael, CA). The number of pups showing the penetrant or silent phenotype in each litter was entered in the statistical model as covariates. mRNA expression data were also analyzed by one-way repeated-measures ANOVA with arcsine data transformation when necessary. When main effects were detected, Holm-Sidak post hoc tests were used to make comparisons with the control group. All statistical tests were performed using the *SigmaStat* (Jandel Scientific, San Rafael, CA) package.

Results

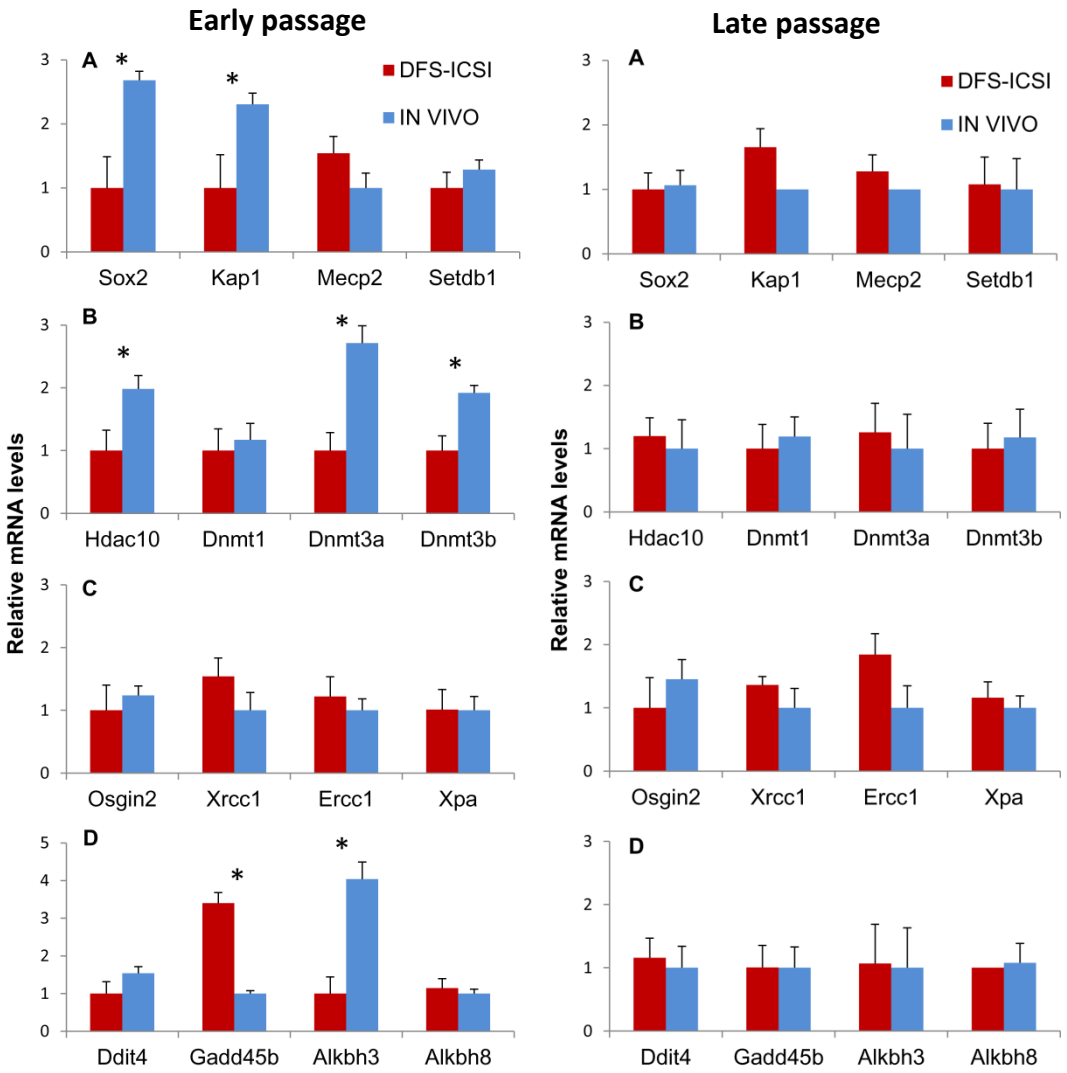
ICSI using DNA-fragmented Sperm Reduces Embryonic Stem Cell Derivation Efficiency without affecting the Karyotype

To examine the effect of DFS-ICSI on ESC derivation, 31 DFS-ICSI-derived blastocysts and 30 control *in vivo*-derived blastocysts were plated onto feeder layers of MEFs in the presence of GSK3β and MEK-inhibitors until the establishment of ESC lines. On Day 4 after seeding on MEFs, we observed the attachment and proliferation up to Day 7 of 10 DFS-ICSI-derived cell clumps and 27 *in vivo*-derived cell clumps, that were trypsinized and passed onto new MEF-coated plates to establish ESC lines. Three days after passaging, 7 DFS-ICSI-derived ESC lines and 25 *in vivo*-derived ESC lines were obtained indicating an efficiency of 83% for the *in vivo*-derived ESC lines and 23% for the DFS-ICSI-derived ESC lines. Both groups of ESC lines were then subject to chromosome analysis. No karyotype abnormalities were detected and appropriate chromosome numbers were observed in 81% (170/209 metaphase spreads) of metaphase spreads prepared from DFS-ICSI-derived ESC lines and 78% (117/150 metaphase spreads) from *in vivo*-derived ESC lines.

Gene Expression in DFS-ICSI-Produced Embryonic Stem Cells

To compare the DFS-ICSI-derived and *in vivo*-derived ESC lines, gene expression profiles were examined upon early passage (passage 0) and after several passages (passage 10). The genes selected were *Sox2*, *Kap1*, *Mecp2* and *Setdb1* due to their known roles in pluripotency and epigenetic repression. The profiles obtained indicated that *Sox2* and *Kap1* were significantly downregulated in the early passage DFS-ICSI-derived ESC lines compared to the *in vivo*-derived ESC lines (Figure 1A).

The expression of genes involved in DNA methylation and histone acetylation, *Hdac10*, *Dnmt3a* and *Dnmt3b*, was also significantly lower in the early passage DFS-ICSI-derived than the *in vivo*-derived ESC lines (Figure 1B). In contrast, no significant differences were detected between the two ESC groups in their expression patterns of *Osgin2*, *Ercc1*, *Xrcc1* and *Xpa* as markers of oxidative stress, base excision repair (BES) and nucleotide excision repair (NES) (Figure 1C). Finally, we assessed DNA damage and repair by analyzing *Ddit4*, *Gadd45b*, *Alkbh3* and *Alkbh8* expression. Significant upregulation was observed of *Gadd45b* and downregulation of *Alkbh3* in the DFS-ICSI-derived ESC lines upon early passage (Figure 1D). No differences were detected in the expression profiles of the late passage cell lines (Figure S1).



Figures 1 and S1. mRNA expression in DFS-ICSI- and *in vivo*-derived ESC lines at early passage (passage 0) and at late passage (passage 10). (A) pluripotency and epigenetic repression genes; (B) DNA methylation and histone acetylation genes; (C) oxidative stress, base excision repair (BES) and nucleotide excision repair (NES) genes; and (D) DNA damage and repair genes. * indicates statistical differences for each gene transcript at P≤0.05; error bars represent SEM.

Effect of ICSI using DNA-fragmented Sperm on the Sperm Count and Motility

No significant differences were recorded in the average weight of either testes or epididymis in the DFS-ICSI-produced and control male mice. However, four DFS-ICSI-produced males (20%) (N=20 mice analyzed) showed a very low testes weight (<0.07 g vs 0.20 g in both WT and rest of ICSI mice), and morphological abnormalities (atrophied testes without presence of spermatozoa). In addition, in three of the DFS-ICSI animals (15%), testes weight was high (≥0.26 g; above the 90th percentile for the colony). In contrast, the control males all showed a normal testes weight and normal testicular morphology (N=20). Significant differences (P=0.002) were detected in the mean number of sperm collected from the cauda epididymis and deferent conduct (6.77± 1.2x 10^6 spz/ml for N=8 DFS-ICSI males versus 15.61± 2.2 x 10^6 spz/ml for N=7 control males). No differences were observed between the DFS-ICSI and control groups in overall sperm motility (64.25% and 72.79% respectively) and progressive sperm motility (32.61% and 32.75% respectively).

Effect of ICSI using DNA-fragmented Sperm on the Copulation and Fertility Rates of the Male Progeny

Pregnancy rates recorded in the B6D2F1 female mice partnered with DFS-ICSI-produced males were significantly reduced compared to the rates observed in females mated with control males (49.96% ±5.24, N = 237) vs. 86.52% ±3.13, 138), Table 1). Moreover, 100% of the females with vaginal plugs mated with control mice became pregnant yet pregnancy was only observed in 68.48% of the females with vaginal plugs mated with DFS-ICSI males. In DFS-ICSI males, a statistically significant decrease in pregnancy rates related to the age was observed. Thus, the ageing phenotype previously observed in these animals [5] could affect fertility as well. No differences were detected in litter size between the DFS-ICSI and control groups. However, significantly higher percentages of pregnant females with resorptions or mummified fetuses were recorded for the DFS-ICSI males. Furthermore, a statistically significant increase in the percentage of pregnant females with resorptions was observed in old males from the control group (Table 1). Some authors have described a decreased reproductive potential (in natural conception, *in vitro* blastocyst development, and implantation potential) during ageing [23] . Although old males from the control group showed high pregnancy rates, it could be possible that aged sperm that should not fertilize yields a high number of resorptions. On the contrary, this difference could not be found in old males from the DFS-ICSI group, as resorption rates were high at all ages. In addition, more than 15% (8/53) of the males in the DFS-ICSI group could be considered infertile since no pregnancies were recorded in response to their partnering with at least 6 virgin females. In a subsequent examination of the testes of these animals, we observed reduced testis sizes and low amounts of sperm.

Table 1: Results of matting of DFS-ICSI and control male mice at 4-6 months (young), 10-12 months (adult), and 16-18 months (old) of age.

Male group	Age (N)	No. of Females	No. vaginal plugs	Pregnant females (%)	Average of puppies by litter	Total No. of resorptions (females %)
Control	Young (16)	45	43	43 (89,6±3,7)[a]	7,7	6 (13,8±5,2)[a]
	Adult (10)	48	38	38 (86,7±7,1)[a]	7,92	5 (12,9±6,5)[a]
	Old (14)	45	39	39 (83,3±5,3)[a]	6,6	14 (36,8±6.1)[b]
	Total (40)	138	120	120 (86,5±3,1)[a]	7,4	25 (21,2±3,7)[a]
DFS-ICSI	Young (16)	48	34	29 (60,4±9,7)[b]	8,17	12 (41,4±11,1)[bd]
	Adult (23)	138	83	65 (47,1±7,6)[c]	7,06	31 (47,7±8,7)[cd]
	Old (14)	51	29	19 (37,2±10,5)[d]	7,8	7 (36,8±12,6)[bd]
	Total (53)	237	165	113 (49,9±5,2)[c]	7,6	50 (36,2±5,9)[bd]

Data are mean ± SEM. Within rows, values followed by different superscript letters differ significantly (P≤0.05)

Histology and TUNEL Labeling of Testicular Tissue Sections

In hematoxylin-stained cross-sections of the seminiferous tubules of adult mice produced by DFS-ICSI, we observed that the mice with small testes (approximately 20%) had evident signs of abnormal spermatogenesis and abnormal tubule morphology. In addition, we noted a lack of germ cells in the atrophied tubules indicating subfertility or infertility (Figure 2B). When we examined 614 control tubule cross-sections (N=8), it was noted that 24.1±3.2% of the sections showed reduced spermatogenesis and an abnormal tubule morphology (Figure 2A). In contrast, out of 1682 tubule cross-sections examined in the DFS-ICSI group (N=8), reduced spermatogenesis was observed in 43.3±3.1% and an abnormal morphology in 14.4± 3.9% (P=0.002) (Figure 2C).

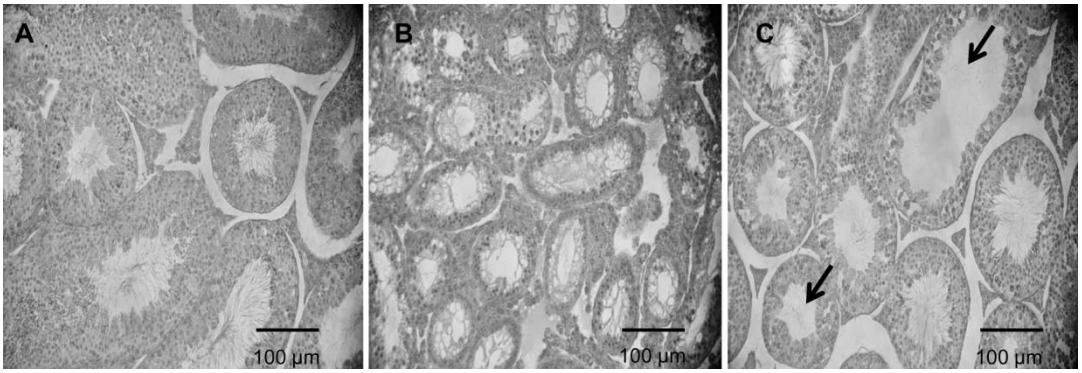


Figure 2. Histological comparisons between seminiferous tubules in adult offspring produced by DFS-ICSI or natural mating. (A) Seminiferous tubules in control mice show a normal shape with germ cells organized in concentric layers and exhibit ongoing germ cell production. (B) Abnormal seminiferous tubules observed in the testes of infertile DFS-ICSI-produced males (20% of the DFS-ICSI male mice with small testes); note their irregular shape and loss of germ cells in many atrophied seminiferous tubules. (C) The testis of an DFS-ICSI male showing reduced fertility (80% of DFS-ICSI males) containing both tubules showing a normal appearance and ongoing spermatogenesis as well as severely degenerated tubules, which are either empty or have only a small germ cell population (arrows).

The TUNEL assay was performed on paraffin-embedded cross-sections of testicular tissue obtained from DFS-ICSI (N=7) (Figure S2C, D) and control (N=5) (Figure S2A, B) adult male mice. At least 200 seminiferous tubules were examined per animal. Our results indicate that compared to control mice, the testes of DFS-ICSI males showed more TUNEL-positive cells/cross-sectioned tubule (apoptosis index) and more spermatogenic cells undergoing apoptosis (Figure S2E).

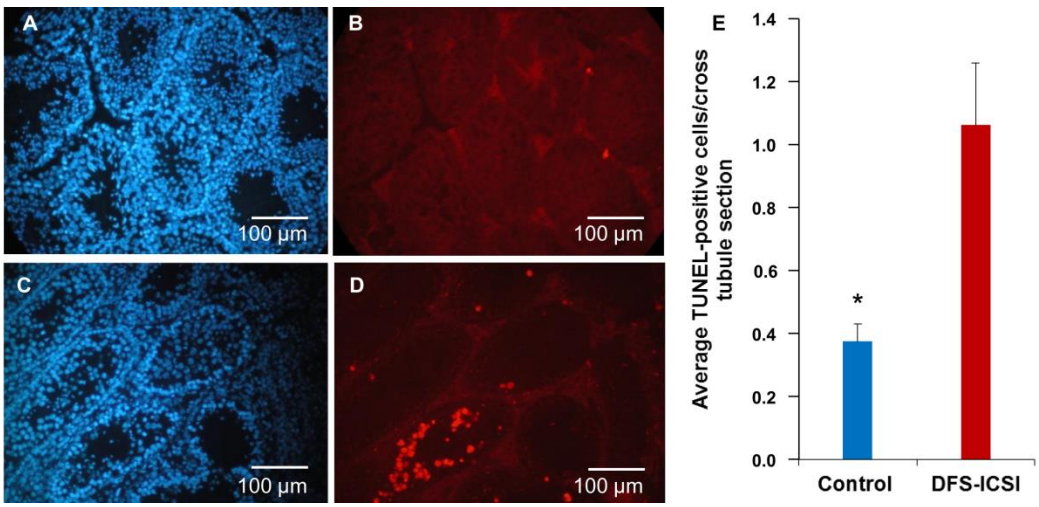


Figure S2. TUNEL analysis in testes from DFS-ICSI- and *in vivo*-produced mice. TUNEL labeling of a cross-section of testes from *in vivo*- (A,B) and DFS-ICSI-produced (C,D) mice. (E) The mean number of TUNEL-positive cells/tubule cross section was higher in the testes of DFS-ICSI-produced mice than control testes. *P<0.05; error bars represent SEM.

ICSI using DNA-fragmented sperm affects the Postnatal Expression of an Epigenetically Labile Allele, Axin1^{Fu}

The *Axin1*$^{Fu/+}$ progeny of oocytes fertilized by DFS-ICSI (N=90) were more likely to have a kinky tail compared to either of the two control groups (*Axin1*$^{Fu/+}$ 2-cell transfer group (N=100) and natural mating group (N=125) (P<0.01, Figure 3). Both control groups also differed (P< 0.05) according to their no kinks or slightly kinky tail phenotype distributions (Figure 3). Our results indicate that the DFS-ICSI fertilization of oocytes led to the birth of more pups that expressed an active *Axin1*$^{Fu/+}$ epiallele, resulting in more pups with a kinky tail. This could not be attributed to the different survival of embryos of a given genotype, nor was it the consequence of superovulation, culture for 24 h until the 2-cell embryo stage or embryo transfer to a pseudopregnant recipient dam.

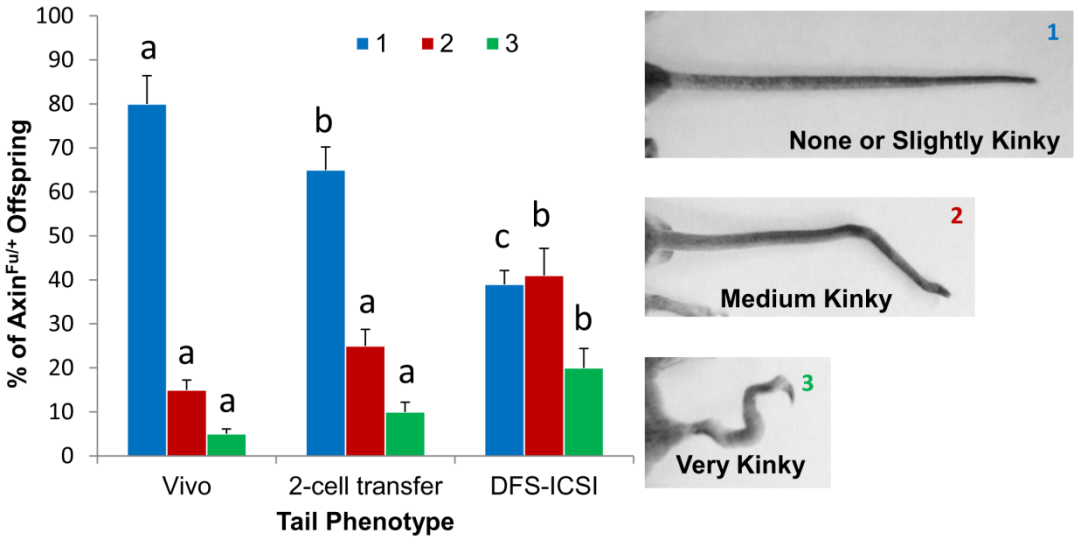


Figure 3. Effect of DFS-ICSI on the tail kinking phenotype of the offspring. Tail phenotypes (1: none or slightly kinky; 2: medium kinky; 3: very kinky) recorded in all the *Axin1*^{F}/+ offspring in the groups: *in vivo*-produced controls, oocytes fertilized *in vivo*, cultured for 24 h and transferred at the 2-cell stage, and DFS-ICSI-fertilized oocytes transferred at the 2-cell stage. DFS-ICSI led to a kinkier tail phenotype. Error bars represent mean ± SD. Bars with different lowercase letters (a, b, c) represent significant differences for each phenotype (P≤0.01).

Discussion

In our model of ICSI we used frozen-thawed sperm because we have previously observed that freezing in the absence of a cryoprotectant gives rise to DNA-fragmented spermatozoa (DFS) with double-strand DNA breaks [5]. In effect, when ICSI is used in human assisted reproduction to overcome male infertility, sperm with damaged DNA incapable of oocyte fertilization *in vivo* are used for fertilization *in vitro*. ICSI with DNA fragmented sperm produces genetic and epigenetic alterations in preimplantation embryos that affect the phenotype of the offspring, and lead to the long term manifestation of a variety of deleterious phenotypes in mice [5]. Here, we used ESCs derived from embryos produced by DFS-ICSI to examine the effects of DFS-ICSI without the need to produce animals. Our data indicate that DFS-ICSI-produced embryos show a reduced potential to generate ESC lines compared to *in vivo*-produced embryos, and that during early passages these ESCs differ in their expression of certain genes. Once passaged 10 times, however, such gene expression differences were lost, confirming the idea that mouse ESCs of different origins will eventually adopt a similar gene and protein expression profiles after several passages [24,25]. The reduced derivation efficiency of ESC lines produced by DFS-ICSI could be a consequence of the low quality of these embryos both in terms of genetics and epigenetics, confirming the low rates of successful implantation and fetal development observed for these embryos [18]. Several studies have tried to correlate sperm DNA integrity with embryo quality and long term effects. Thus, 40% of embryos generated by ICSI using DFS showed abnormal chromosome segregation and chromosome fragmentation; and half of these embryos with abnormal chromosomes developed into normal-looking blastocysts and were capable of implantation. However, almost all of them aborted spontaneously before embryonic Day 7.5 [26]. The reduced number of ESC lines derived here from our DFS-ICSI embryos and the fact that all the lines showed a normal karyotype suggests that embryos with an abnormal karyotype are unable to produce ESC lines.

Our observation of gene expression differences between early passaged DFS-ICSI-derived and *in vivo*-produced ESC lines including the down-regulation of both *Hdac10* and the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* in the DFS-ICSI-derived ESC lines may confirm epigenetic differences among DFS-ICSI-generated embryos [5]. Hypomethylation in ESCs has been also linked to the down-regulation of *Dnmt3a* and *Dnmt3b* and to differentiation dissimilarities [27]. Moreover, the lower expression of *Sox2* observed here could be related to the reduced pluripotency of these ESCs. Also, the transcriptional repression of *Kap1* along with *Sox2*, *Hdac10*, *Dnmt3a*, and *Dnmt3b*, as modifiers of epigenetic gene silencing through the transcription of specific genes, involves changes in chromatin state. Interestingly, these genes are retained in the small fraction of sperm

DNA bound by nucleo-histones [28,29] suggesting that the mechanism whereby DFS-ICSI modifies phenotype could be related to mechanisms of epigenetic gene silencing. The higher expression of *Gadd45* in our DFS-ICSI-derived ESC lines may support some sort of DNA damage in these cells. In addition, *Gadd45* family proteins have been attributed a role in senescence and aging, and this phenotype is typical of DFS-ICSI-generated mice [5]. The reduced expression of the repair enzyme-coding *Alkbh3* [30] detected in our DFS-ICSI-derived ESC lines is in agreement with the premature aging of DFS-ICSI-generated mice [5].

When we examined the effects of DFS-ICSI on male fertility, we identified a group of mice showing small testes and infertility that represented 20% of the male animals produced. Remaining DFS-ICSI males with normal-sized testes showed reduced average fertility during all stages of life. Other studies have analyzed the effect of ICSI with normal sperm on fertility. When gene expression in the testes of both ICSI-produced and naturally conceived mice by micro-array analysis was examined, 474 (150 up-regulated and 324 down-regulated in ICSI mice) differentially expressed genes were identified representing several functional pathways, including those implicated in spermatogenesis, male meiosis I, spermatid development, gonad development and male genitalia development. It has been observed that such differential gene expression patterns are transmitted to the next generation [31]. It has been also reported that ICSI-derived mice exhibit a high level of spermatogenic cell apoptosis, suggesting a risk of the compromised fertility of male progeny [32]. Our observation of the reduced *in vivo* fertility of DFS-ICSI male mice is consistent with such findings and indicates that DFS-ICSI could compromise the fertility of the male offspring in other mammals.

In this study, we also examined the effects of DFS-ICSI on the postnatal expression of an epigenetically labile allele, *Axin1^{Fu}*. Our results indicate that DFS-ICSI perturbs the epigenetic reprogramming of *Axin1^{Fu}* causing a shift towards the active state of the epiallele. The manifestation of this was the birth of more pups expressing the active epiallele rendering a kinky tail phenotype. Similar results have been reported for the effect of 4 days of IVC on the *Axin1^{Fu}* and *A^{vy}* alleles [33]. Collectively, our results suggest that such effects of ICSI and IVC are likely to affect metastable epialleles in general and reveal that while primary epimutations produced by ICSI in mice can be properly corrected in the germ line by epigenetic reprogramming [7], the alterations produced by ICSI in some metastable epialleles like *Axin1^{Fu}* are propagated to subsequent generations. With regard to the question of whether the rest of the genome may have mechanisms similar to the regulation of the *Axin1^{Fu}* allele, we know that transposable elements represent up to 45-50% of mouse and human genomes [8]. Many of these new alleles produced by the insertion of a transposable element are expressed during

preimplantation development [34]. Given the conservation of epigenetic mechanisms during evolution in mice and humans, it is likely that similar mechanisms of metastable epialleles will be active in humans. Our results point to the notion that changes in the epigenetic state of the genome can be induced early in development by environmental conditions, and that these changes can have consequences for both gene expression in adulthood [35,36] and the inheritance of epigenetic phenotypes. We are unaware of the reason why DFS-ICSI renders a kinkier tail phenotype, though a kinked tail has been described as an embryopathy produced by oxidative DNA damage due to ROS [37]. One of the targets for oxidative DNA damage is the methylated base m^5C found in mammalian DNA. Thus, if DFS-ICSI increases ROS levels in the embryo, this may produce oxidative DNA damage and preferentially affect m^5C.

In humans, ICSI is currently used as a successful infertility treatment. Although a significantly increased risk of birth defects in infants conceived by assisted reproductive technology (ART) has been described in the last years, it has been reported that there is no risk difference between children conceived by IVF and/or ICSI [38]. Some studies have suggested that this increased risk may be due to the underlying infertility of the couples pursuing ART, and not to ARTs themselves [38,39,40]. Furthermore, significant limitations of human studies are the lack of a good comparison group for IVF or ICSI, which would be babies naturally conceived by infertile couples rather than babies conceived by overall population, and the low power of the studies due to the rarity of the diseases [39]. However, a potential risk of ICSI is the use of spermatozoa with apparently normal morphology but with DNA fragmentation. A significant proportion of infertile men have elevated levels of DNA damage in their ejaculated spermatozoa [41]. Sperm DNA damage is a useful biomarker for male infertility diagnosis and it is associated with reduced fertilization rates, embryo quality and pregnancy rates, and higher rates of spontaneous miscarriage and childhood diseases [42]. It remains unclear whether assisted reproductive techniques can compensate for DNA damage. Hence, studies conducted with animal models are particularly important. Some of the effects described in our manuscript regarding male infertility in DFS-ICSI mice have been previously described in mice produced by ICSI with intact fresh sperm. Yu *et al.* reported decreased testis weight, abnormal tubule morphology and increased apoptosis in testis of adult mice produced by ICSI with fresh sperm [32]. Other studies with ICSI using fresh sperm described diverse alterations as transcriptome perturbations that remained at the neonatal stage [43], or alterations in glucose parameters in adult mice [44]. Studies that induce sperm DNA damage and evaluate its biologic effects on the offspring and on next generations are of vital importance. To date, few animal studies have assessed the effects of induced sperm DNA damage on fertilization and embryo development. Yamagata *et al.* described abnormal chromosome segregation in embryos generated by ICSI with fresh sperm or

with DFS. We have previously described that more severe abnormalities appear when ICSI is performed with DFS compared to ICSI performed with fresh sperm. Although there were no differences in fertilization and embryo developmental competence, pregnancy rates, live offspring rates and survival after 25 weeks were significantly lower. Furthermore, ageing phenotype and tumor development were observed in DFS-ICSI animals but not in animals generated by ICSI with fresh sperm [5]. Thus, from these data we could speculate that although ICSI *per se* (performed with fresh sperm) can produce several alterations, abnormal phenotypes are more severe when ICSI is performed with DFS. However, comparative analyses of ICSI with fresh sperm *vs.* DFS-ICSI are very important.

Our findings offer new motives for current concerns over the safety of ICSI with DFS. Especially worrying is the frequent use of ICSI in cases of severe male factor infertility, since a significant proportion of the spermatozoa used for ICSI are likely to have fragmented DNA. The subacute nature of some of the aberrant embryo modifications induced by ICSI with DFS means that many of these changes will be undetected in the short term. Moreover, embryo development to the blastocyst stage, considered a hallmark of ART system efficiency, is often possible despite detrimental environmental effects and longer term consequences of this procedure [5]. Our first conclusion is that we should only use early passage DFS-ICSI-derived ESC lines to assess specific alterations associated with the DFS-ICSI technique. Secondly, we demonstrate here that DFS-ICSI in the mouse modifies reprogramming in a manner that favors the active state of the *Axin1*^{Fu} epiallele, and that this epigenetic alteration is transmitted to the following generations. Thirdly, our findings also reveal a dramatic influence of DFS-ICSI on the reproductive lifespan of male progeny. This could have significant implications for the reproductive management of livestock, endangered species and also humans.

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Germ Cell culture conditions facilitate the reprogramming to produce mouse ESCs

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Chapter II / Capítulo II

Summary

Different cell types have been suggested to be the origin of ESCs since they were first isolated. One of the solidest theories proposed that ESCs could arise from epiblast cells that are already predisposed to a primordial germ cell (PGC) fate. In the same way, many culture conditions for ESCs derivation have been developed that show different results. The objective of this chapter was to determine the effect of a germ cells culture (GS) medium during ESCs derivation. To do so, differential cell counting in blastocysts produced in different culture conditions, ESCs derivation efficiency, karyotype, chimaera generation ability and expression of genes related to pluripotency, germline and epigenetics were analyzed. It was observed that LIF supplementation during embryo culture increased the proportion of ICM cells. Thereafter, blastocysts cultured in GS medium increased ESCs derivation efficiency, showing normal karyotype and higher chimera formation capacity by blastocyst ESCs microinjection. ESCs lines derived in GS medium showed an appropriate expression of pluripotency-related genes, and a correlation was found between derivation efficiency and the expression of some imprinting genes and retrotransposons. Although germ cell-specific genes were expressed in all culture conditions, GS medium did not induce an upregulation of germline-specific markers during the transition from blastocyst to ESCs.

In conclusion, ESCs derivation is conditioned by culture medium, and the combination of LIF supplementation followed by culture in GS medium constitutes a high efficiency method for ESCs derivation.

Resumen

Desde las ESCs que fueron aisladas por vez primera, se han propuesto diferentes orígenes para estas células. Una de las teorías más sólidas plantea que las ESCs podrían proceder de células del epiblasto que estuvieran predestinadas a convertirse en células primordiales germinales (PGCs). Del mismo modo se han desarrollado múltiples condiciones de cultivo para la obtención de ESCs. El objetivo de este capítulo ha sido determinar el efecto de diferentes condiciones de cultivo, y en concreto de un medio de cultivo para células germinales (GS), en la obtención de ESCs. Para ello se hizo recuento celular diferencial en los blastocistos producidos en las distintas condiciones de cultivo y se analizó la eficiencia de obtención de ESCs, el cariotipo, la capacidad de formación de animales quiméricos y la expresión de diferentes genes relacionados con pluripotencia, linaje germinal y epigenética. Se observó que la proporción de células de la ICM aumentó en los blastocistos producidos en medio suplementado con LIF. Posteriormente, el cultivo de los blastocistos en medio GS aumentó la producción de líneas de ESCs, que además mostraban un cariotipo normal y una capacidad superior de formación de animales quiméricos mediante microinyección de ESCs en blastocistos. Las ESCs obtenidas en medio GS mostraron una expresión correcta de genes relacionados con la pluripotencia, y se encontró una correlación entre su eficiencia de obtención y la expresión de algunos genes improntados y algunos retrotransposones. Aunque se detectó expresión de genes relacionados con el linaje germinal en todas las condiciones de cultivo, el medio GS no produjo una mayor expresión de marcadores de linaje germinal durante la transición de blastocisto a ESCs.

En resumen, la obtención de ESCs está condicionada por la utilización de diferentes medios de cultivo, y la combinación de LIF y de medio de cultivo GS constituye un método eficiente para la obtención de ESCs.

Germ Cell culture conditions facilitate the reprogramming to produce mouse ESCs

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Abstract

The derivation of embryonic stem (ES) cell lines from blastocysts is a very inefficient process. It has been proposed that murine ES cells could arise from epiblast cells that are already predisposed to a primordial germ cell (PGC) fate. We have demonstrated that if during the process of ES cell derivation in B6D2 F1 hybrid mice, we first culture the embryo in medium supplemented with LIF we improve the quality of the blastocyst. Thereafter, when blastocyst is cultured in a germ cells culture (GS) media, we are able to obtain good quality ES cell lines with a higher efficiency, showing normal karyotype, increasing the degree of chimerism and germline transmission by ES cells microinjection in blastocysts. Although germ cell-specific genes were expressed in all culture medium conditions, GS medium did not induce a more pronounced shift towards germ cell specification. Furthermore, a correlation was observed between ES cell derivation efficiency and some imprinted genes and retrotransposable elements expression. In conclusion, the combination of LIF supplementation followed by culture in GS medium constitutes a high efficiency method for ES cell derivation.

Introduction

It is remarkable that pluripotent stem cell lines can be derived from preimplantation embryos, since *in vivo* pluripotent cells of the early mammalian embryo only briefly proliferate before differentiating to a more developmentaly restricted cell type. However, certain cells from the blastocyst can be isolated and, under particular culture conditions, give raise to embryonic stem (ES) cells, which can indefinitely self-renew *in vitro* while maintaining an undifferentiated pluripotent state.

Mouse embryonic stem (mES) cells grow as round compact colonies of small cells, which depend on leukemia inhibitory factor (LIF). LIF is a cytokine produced by the endometrium to allow blastocyst implantation (Pera and Tam 2010). The effect of LIF on the *in vitro* development of murine embryos has been widely studied with oftenly contradictory results. In some studies LIF has been demonstrated to enhance mouse blastocyst formation and decrease embryo fragmentation (Tsai et al. 1999; Cheng et al. 2004b), while in other studies LIF in standard medium did not enhance the development of human or bovine early embryos (Jurisicova et al. 1995; Vejlsted et al. 2005). However, these contradictory results may be attributable to the different species and culture systems used for different experiments.

Although early studies indicated that murine ES cell lines were derived from the inner cell mass (ICM), some later experiments suggested that ES cells more closely resemble cells from the epiblast or primitive ectoderm (Brook and Gardner 1997). Thus, using conventional methods, isolated mouse primitive ectoderm gives raise to ES cell lines at a higher frequency than does isolated ICM. Moreover, the culture of primitive ectoderm allows the isolation of ES cell lines from mouse strains that have been previously refractory to ES cell isolation (Brook and Gardner 1997) and naïve ES cell lines can be derived from single, isolated, mouse primitive ectoderm cells, which is not possible with ICM cells (Nichols et al. 2009). However, although these data suggest that ES cells are more closely related to primitive ectoderm than to ICM, they still cannot ascertain whether ES cells directly relate to primitive ectoderm or to a cell derived from it *in vitro*.

Accordingly, gene expression analyses indicate that the closest *in vivo* equivalent to ES cells is an early germ cell, rather than ICM or primitive ectoderm (Zwaka 2005). Recently, bone morphogenetic protein (BMP) signaling has been shown to be important for the self-renewal of mouse ES cells (Ying et al. 2003), whereas *Bmp4* (Lawson et al. 1999) and *Bmp8b* (Ying et al. 2000) are required for primordial germ cells (PGCs) formation. Moreover, alkaline phosphatase is strongly expressed by early germ cells and by ES cells, but is weakly expressed by the epiblast and other surrounding embryonic cells (Chiquoine 1954; Ginsburg et al. 1990). Several early and later germ cell markers have been

described to be expressed in ES cells: *Dppa3* (Saitou et al. 2002), *Ifitm3* (Saitou et al. 2002), c-*Kit* (Horie et al. 1991)*, DAZL* (Clark et al. 2004) and *Ddx4* (Toyooka et al. 2003). Therefore, at least some of the germ cell-specific genes expressed by ES cells, and not by primitive ectoderm cells, are essential for the long-term maintenance of the pluripotent state (Zwaka 2005). Moreover, pluripotent embryonic germ (EG) cell lines have been isolated from PGCs (Resnick et al. 1992; Matsui et al. 1992; Durcova-Hills et al. 2001), being remarkably similar to mouse ES cell lines (Donovan and de Miguel 2003).

Because of the similarities existing between ES cells and germ cells, we decided to supplement ES cell culture medium with growth factors commonly used for germline stem (GS) cell culture (Kanatsu-Shinohara et al. 2003) to increase the efficiency in ES cell line derivation with pluripotency standards. Different factors seem to be relevant in determining the success of ES cell derivation: the mice strain, the *in vivo* or *in vitro* origin of the blastocyst, the protocol for *in vitro* culture up to the blastocyst stage or the medium used for ES cell culture. In the present study we analyzed the effect of LIF supplementation during *in vitro* embryo culture up to blastocyst stage. Subsequently, for ES cell line derivation, blastocysts were cultured in ES or GS medium. With all these conditions we report here the requirements for a better ES derivation efficiency and epigenetic reprogramming during the progress from two cell embryos to ES cell status.

Results

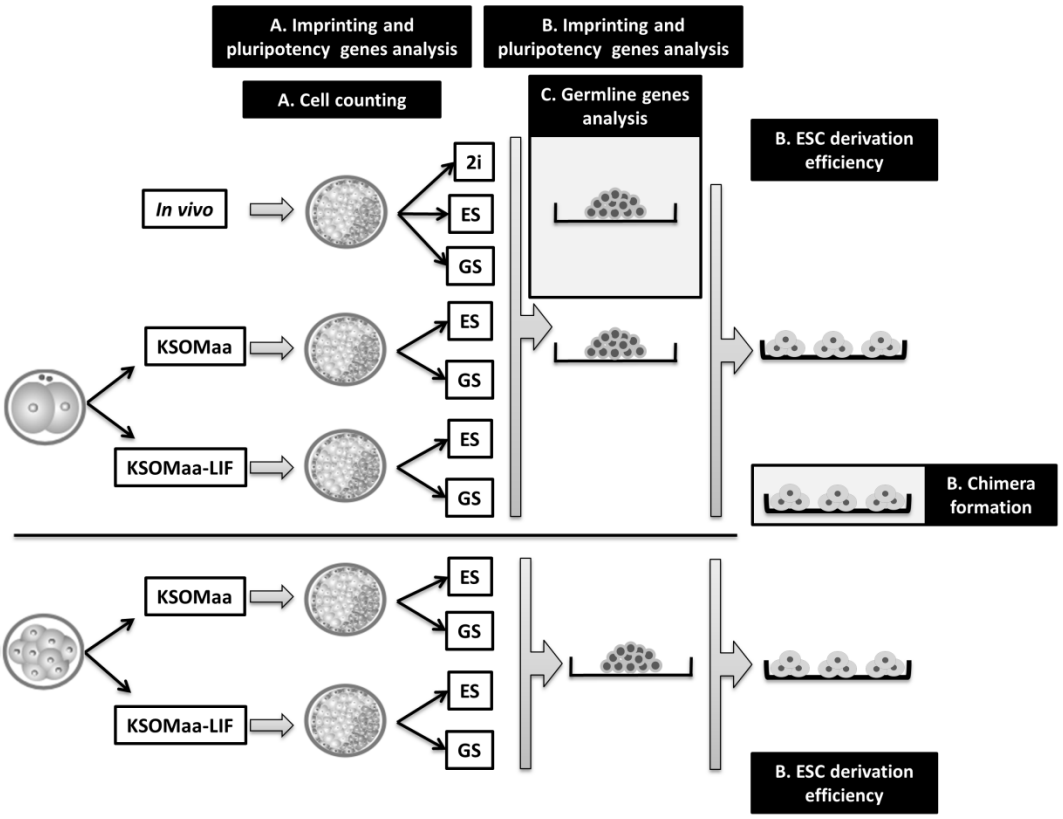

Figure 1. Experimental design.

LIF supplementation increases the ICM/Total cells ratio.

In order to analyze the possible beneficial effect of LIF supplementation in *in vitro* embryo culture at different stages, 2-cell embryos were cultured in KSOMaa or KSOMaa supplemented with LIF up to blastocyst stage (Fig 1). At that point differential cell counting data were compared with that obtained from *in vivo* blastocysts. Embryos cultured in KSOMaa-LIF had a significantly lower total trophectoderm cells number than embryos cultured in KSOMaa or than blastocysts obtained *in vivo* (Fig. 2a). Furthermore, although the blastocysts obtained in the three different conditions did not show significant differences in the total number of ICM cells, those blastocysts cultured with LIF supplementation showed a significantly higher ICM/Total ratio (41,4%) than *in vivo* produced blastocysts (29,3%) and *in vitro* cultured blastocysts without LIF supplementation (27%) (Fig. 2b).

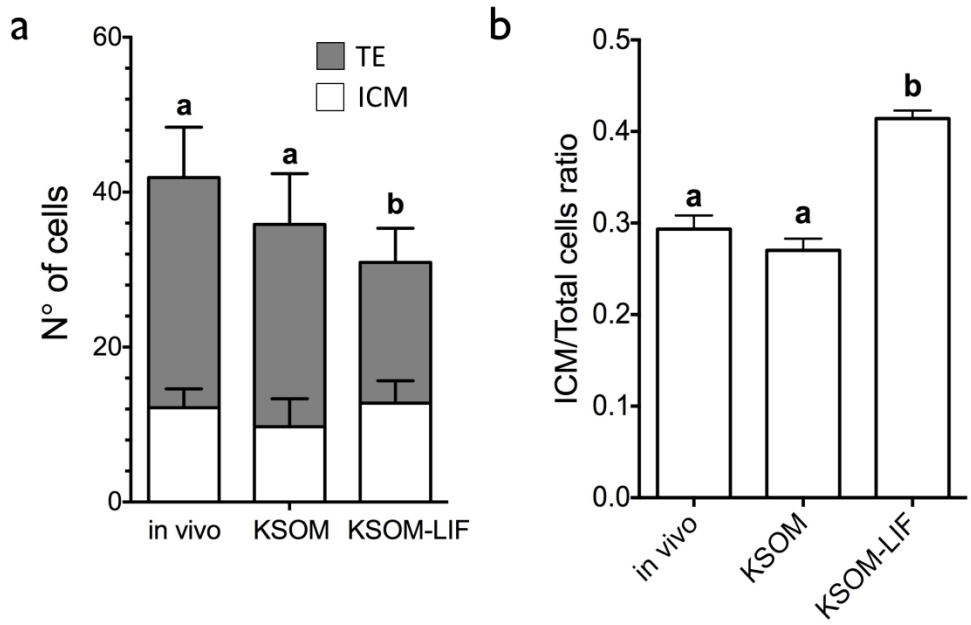

Figure 2. (**a**) Differential ICM (white) and TE (grey) cell counts from *in vivo* blastocysts or blastocysts derived from 2-cell embryos cultured in KSOMaa or KSOMaa-LIF. a, b, indicate $p < 0.05$, Kruskall-Wallis; Dunn's multiple comparison post-hoc test (**b**) Ratio ICM/total cell counts from *in vivo* blastocysts or blastocysts cultured in KSOMaa or KSOMaa-LIF. a, b, indicate $p < 0.0001$, Kruskall-Wallis; Dunn's multiple comparison post-hoc test.

GS culture medium increases the ES cell lines derivation efficiency.

Next, we went on to examine the efficiency of murine ES cell line derivation from *in vitro* or *in vivo* blastocysts cultured in GS medium, and whether LIF supplementation during the culture of two-cell embryos up to blastocysts was complemented by the posterior effect of culturing the blastocysts in GS medium (Fig 1).

Indeed, the efficiency of ES cell line derivation was significantly higher when *in vivo* blastocysts or blastocysts coming from 2 and 8-cell embryos cultured in KSOMaa-LIF were subsequently cultured in GS medium (Table 1). No ES cell line was obtained when two-cell stage embryos were cultured in KSOMaa up to blastocyst, independently of the medium employed thereafter (ES or GS medium). Similarly, when 8-cell embryos were cultured in KSOMaa up to blastocyst ES cell derivation was completely hampered if cultured in ES medium.

ES cells obtained from two-cells KSOMaa-LIF+GS exhibited a normal karyotype (more than 80% of normal metaphases similar to the percentage of the original *in vivo* ES cells) (data not shown).

Table 1. ES cells derivation efficiency in different culture medium conditions.

Embryo culture condition	*ES cells derivation medium*	*No. embryo*	*No. ES cell lines (%)*
***In vivo* blastocysts**	ES	35	3 (8.6%)[a]
	GS	91	12 (13.2%)[b]
2-cell KSOMaa	ES	67	0 (0%)[c]
	GS	48	0 (0%)[c]
2-cell KSOMaa-LIF	ES	55	5 (9.1%)[a]
	GS	60	17 (28.3%)[d]
8-cell KSOMaa	ES	54	1 (1.9%)[c]
	GS	58	1 (1.7%)[c]
8-cell KSOMaa-LIF	ES	65	0 (0%)[c]
	GS	59	10 (16.9%)[e]

[a,b,c,d,e] Different superscript letters indicate significant differences (P = <0,05), One-way Anova, Holm-Sidak method.

Thus, blastocysts cultured in GS medium showed a significantly higher efficiency to derive ES cell lines compared with ES medium culture. Moreover, LIF supplementation during 2 or 8-cell culture up to blastocyst is also a crucial aspect on ES cell derivation.

GS culture medium produces a better epigenetic reprogramming pattern.

In vivo blastocysts or blastocysts obtained by two-cell stage embryos cultured in KSOMaa or KSOMaa-LIF, or their outgrowths, cultured either with ES or GS medium on supportive MEF monolayers for four days, were collected to perform gene expression analysis during the early steps of the ES cells derivation process. No significant differences in pluripotency markers *Nanog*, *Pou5f1* and *Slc2a1* were observed in blastocysts of the different groups performing a second level of normalization of data considering the ICM/total cells (Fig. 3a). Furthermore, when pluripotency markers were analyzed in cell clumps, no significant differences were found in *Slc2a1* expression among the different groups. However, a significantly lower expression of *Pou5f1* was observed in the *in vivo*+ES group (Fig. 3b). *In vivo*+GS and KSOMaa+GS groups showed a significantly higher expression of *Nanog* than *in vivo*+ES and KSOMaa+ES groups. In contrast, KSOMaa-LIF+ES and KSOMaa-LIF+GS groups showed an intermediate expression that was not significantly different with the other groups.

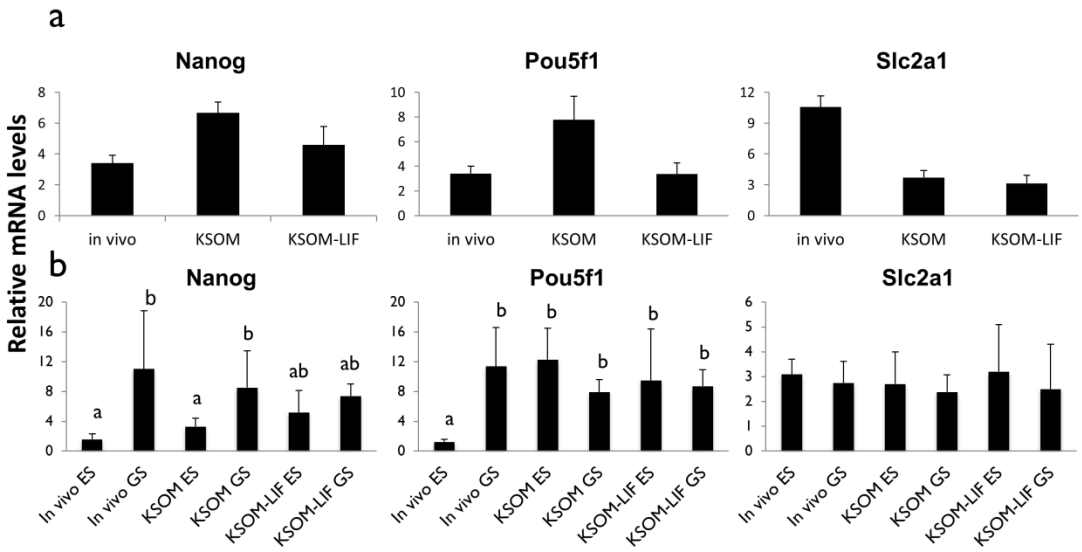

Figure 3. mRNA expression of pluripotency markers. *Nanog*, *Pou5f1* and *Slc2a1* expression in blastocysts, performing a second level of normalization of data considering the ICM/total cells (**a**) or in cell clumps originating from *in vivo* blastocysts or blastocysts (**b**) obtained from two-cells embryos cultured in KSOMaa or KSOMaa-LIF and thereafter in ES or GS medium. a, b, indicate $p < 0.05$, 1-way ANOVA; Holm-Sidak post-hoc test.

Since a significantly higher ES cell derivation efficiency was observed when GS medium was used, we analyzed germline-specific genes in cell clumps derived from *in vivo* blastocysts cultured in ES, GS and 2i media, which has also been demonstrated to enhance ES cell derivation efficiency (Ying et al. 2008) (Fig 1).

Early germline markers expression was detected in all culture conditions. No significant differences were found in *Alk2* expression among the different culture media, while *Bmp4* was overexpressed in the ES group. Posterior germline marker *Ifitm3* (*Fragilis)* was significantly upregulated in GS culture medium compared to ES and 2i culture media, and *Dppa3 (Stella)* was upregulated in the 2i group. Finally, late germline marker *Ddx4* expression was significantly higher in ES culture medium than in GS and 2i culture media (Fig 4).

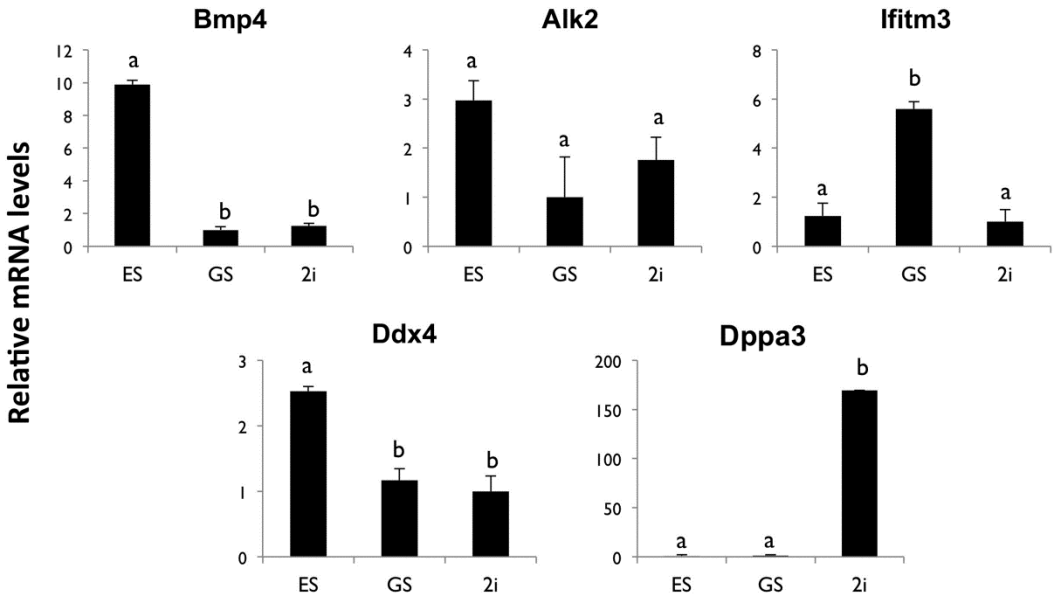

Figure 4. mRNA expression of genes controlling germline establishment. *Bmp4*, *Alk2*, *Ifitm3, Dppa3* and *Ddx4* markers expression in cell clumps from *in vivo* blastocysts cultured in ES, GS or 2i media. a, b, indicate $p < 0.05$, 1-way ANOVA; Holm-Sidak post-hoc test.

It has been demonstrated that a few transcripts encoded by the imprinted *Dlk1-Dio3* gene cluster, including *Meg3* (also known as *Gtl2*), are aberrantly silenced in iPS cell lines with poor contribution to chimaera (Stadtfeld et al. 2010). Consequently, we analyzed *Meg3* expression as a quality indicator in our ES cell lines. Quantitative PCR (qPCR) analysis of *Meg3* confirmed that GS culture conditions increase the expression of *Meg3*. Thus, KSOMaa-LIF+GS group showed a significantly higher *Meg3* expression than *in vivo*+ES, KSOMaa+ES, KSOMaa+GS and KSOMaa-LIF+ES groups (Fig. 5a). Furthermore, a correlation was found between the efficiency to obtain new ES cell lines and *Meg3* expression levels (spearman r 0.7827) (Fig. 5b).

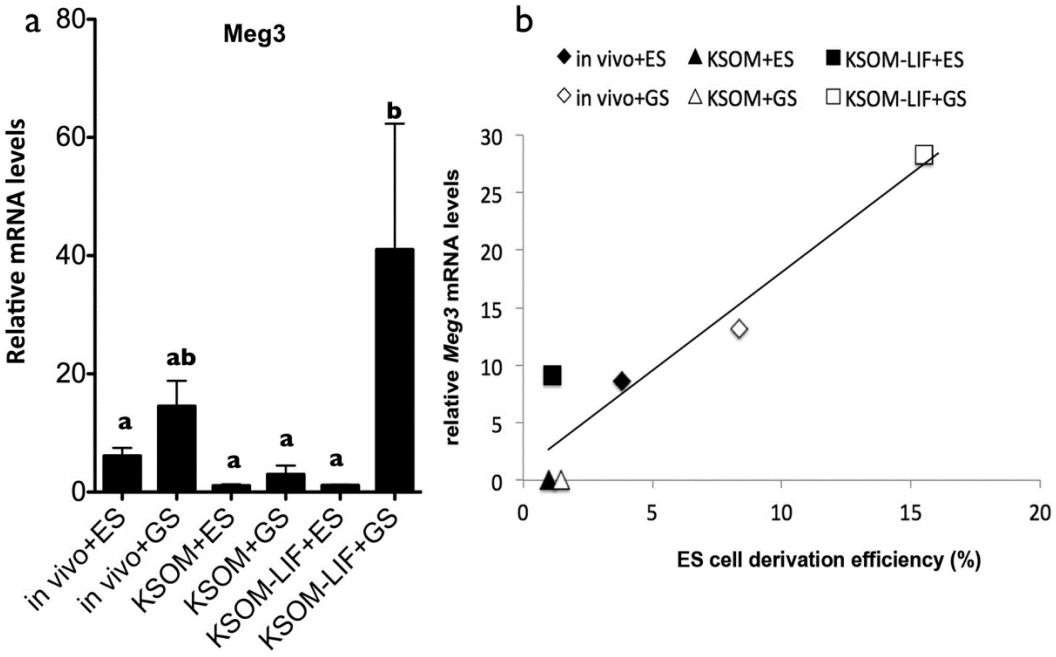

Figure 5. *Meg3* expression. (a) *Meg3* marker expression from cell clumps originating from *in vivo* blastocysts or blastocysts obtained from two-cells embryos cultured in KSOMaa or KSOMaa-LIF. *P < 0.05, 1-way ANOVA; Holm-Sidak post-hoc test. (**b**) Correlation between the efficiency of ES cell derivation in different culture conditions and the expression levels of *Meg3* marker. Spearman correlation. r = 0.7827.

We also found significant differences in other imprinted genes expression levels such as *Rhox5*, *U2af1-rs1*, and in the retrotransposable element *IAP* (Fig. 6). *In vivo*+ES, *in vivo*+GS and KSOMaa-LIF+GS groups showed a significantly lower *Rhox5* expression than KSOMaa+ES, KSOMaa+GS and KSOMaa-LIF+ES groups (Fig. 6). *U2af1-rs1* and *IAP* expression in *in vivo*+ES and *in vivo*+GS groups was significantly lower than in KSOMaa+ES, KSOMaa+GS and KSOMaa-LIF+ES groups.

These expression analyses further confirmed that GS medium and the addition of LIF during blastocyst culture produce a better pluripotency pattern and a higher expression of *Meg3* that correlated with ES derivation efficiency.

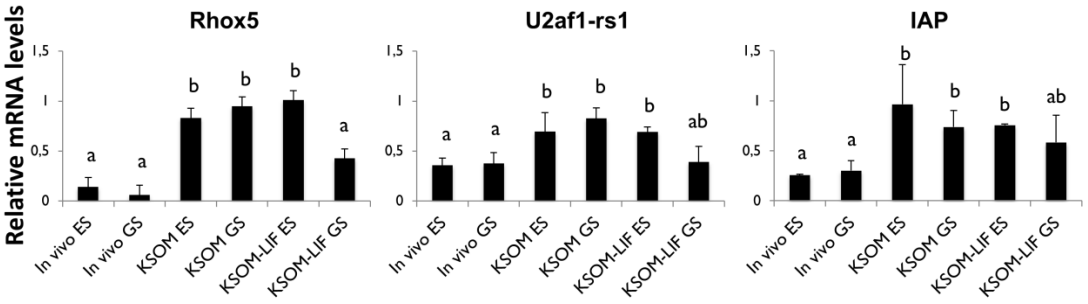

Figure 6. mRNA expression of imprinted genes. *Rhox5*, *U2af1-rs1* and *IAP* expression from cell clumps originating from *in vivo* blastocysts or blastocysts obtained from two-cells embryos cultured in KSOMaa or KSOMaa-LIF and later in ES or GS medium. a, b, indicate $p < 0.05$, 1-way ANOVA; Holm-Sidak post-hoc test.

ES cells from KSOMaa-LIF+GS medium culture showed an increased chimerism capacity

We next studied the ability of chimera formation in different ES cell lines. We analyzed three different ES cell lines obtained from the three *in vitro* conditions which showed a significantly higher derivation efficiency: 2-cell KSOMaa-LIF+ES (ES/ES(2)2 cell line); 2-cell KSOMaa-LIF+GS (ES/GS(2)7 cell line) or 8-cell KSOMaa-LIF+GS (ES/GS(8)4 cell line) (shaded rows in Table 1). The ES cell line ES/GS(2)7 showed a significantly higher percentage of mice with more than 75% of ES-cell coat color after injection in blastocysts, compared to ES/ES(2)2 (35.7% vs 3.8%) (Table 2). Furthermore, germline transmission was also higher in chimeric mice with more than 75% ES-cell coat color obtained from ES cell line ES/GS(2)7 injection (Fig 7a) than in those produced by ES/ES(2)2 (Fig 7b) (100% vs 14.3% respectively).

Figure 7. Germline transmission in chimeric mice from ES/GS(2)7 cell line (**a**) or ES/ES (2)2 cell line (**b**), with more than 75% ES cell coat color.

Table 2. Effect of ES cells origin in the production of ES cell mice from ES cells injected into blastocysts

ES cells	Injected embryos	Transferred embryos	Recipient mice	Newborns (%)	Total chimeras (%)	Males with more of 75% ES cell coat color	Males with 100% ES cell coat color
ESGS(2)7	103	91	5	14 (15.4%)	5 (35.7%)	5 (35.7%)[a]	1 (7.1%)
ESGS(8)4	45	42	2	13 (30.9%)	1 (7.7%)	0 (0%)[ab]	0 (0%)
ESES(2)2	108	102	5	26 (25.5%)	3 (11.5%)	1 (3.8%)[b]	0 (0%)

Values followed by different superscript letters differ significantly ($p < 0.05$), Z test with Yates correction.

Discussion

In this study we investigated the effect of LIF supplementation on the *in vitro* culture of murine embryos destined to derive ES cells, as well as the addition of germline-related factors in the culture medium used for ES cell derivation, in order to improve its efficiency and quality.

LIF has been demonstrated to play an important role in *in vivo* embryo development and implantation in mice (Stewart et al. 1992). However, contradictory information exists regarding the effect of LIF in embryo development *in vitro*. Some studies have reported that LIF increases blastocyst formation rates in mice and human (Dunglison et al. 1996; Tsai et al. 2000; Rungsiwiwut et al. 2008), while other reports showed no differences in the development efficiency of human embryos (Jurisicova et al. 1995). Rungsiwiwut *et al*. observed an increment in the total cell number of the blastocysts after KSOMaa-LIF culture in mice (Rungsiwiwut et al. 2008). However, no differential ICM/TE cell number counting was performed in any of the preceding articles. In our study, although KSOMaa-LIF cultured embryos showed a lower total cell number compared to *in vivo* and KSOMaa cultured blastocysts, the ratio of ICM/total cells was significantly higher than in the other groups, which indicates the importance of performing a differential cell count. These blastocysts proved to be more suited for ES cell derivation, as they showed a higher EScell derivation efficiency.

In addition, the highest ES cell line derivation efficiency was attained when embryos were first cultured in KSOMaa-LIF, followed by culture in GS medium. There may be a synergy between LIF supplementation during *in vitro* embryo culture and the posterior addition of other mouse growth factors present in the GS medium ie. EGF, GDNF and bFGF (Kanatsu-Shinohara et al. 2003), that favors the establishment of new ES cell lines. ES-like cells have been established *in vitro* from neonatal gonocytes and adult Spermatogonial Stem Cells (SSCs) in the presence of EGF, GDNF, bFGF and LIF (Kanatsu-Shinohara et al. 2003; Kanatsu-Shinohara et al. 2004). These factors are also known to affect the proliferation and maintenance of other stem cell populations: EGF enhances tissue regeneration in various adult organs such as skin, liver and intestinal epithelium (Reynolds and Weiss 1992); GDNF has been shown to stimulate SSC self-renewal *in vivo* (Meng et al. 2000), and bFGF and LIF are necessary for EG cell derivation and culture, being these cell lines remarkably similar to ES cell lines (Donovan and de Miguel 2003).

Our overall performance was lower than that reported in the literature in recent years, probably because we have employed the standard ES cell derivation method (Evans and Kaufman 1981) that supplements culture medium with fetal bovine serum (FBS), which shows variations between different batches. A defined media supplement, knockout

serum replacement (KSR) with knockout DMEM (KSR-KDMEM) has been shown to facilitate the generation of ES cell lines, yielding higher efficiency for inbred mouse strains ES cell derivation in comparison to DMEM with FBS (Cheng et al. 2004a). More recently, the use of serum-free N2B27 medium supplemented with small-molecule inhibitors of MEK (2i medium) [MAPK (mitogen activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] and GSK3β (glycogen synthase kinase 3β) proved to be very efficient for ES cell derivation (Ying et al. 2008). Future experiments would have to assess whether our implementations provide further efficiency enhancement when combined with these optimized media.

The efficiency of ES cell derivation is greatly strain dependent. Conventional methods for deriving ES cell lines are suitable for mouse strain 129, but not for other refractory strains, so that very few ES cell lines are currently available from inbred strains (mostly C57BL/6). In addition, ES cells derived from C57BL/6 blastocysts are more difficult to propagate *in vitro*, less efficient for chimera generation, and contribute less frequently to the germline (Brook and Gardner 1997; Auerbach et al. 2000). In the present study, we derived ES cell lines from CBAxC57BL/6 hybrid mice, which are widely used in genetic studies.

Although no differences were observed in *Nanog* expression during blastocyst stage, higher expression was observed in outgrowths coming from blastocysts cultured in GS medium. *Nanog* is considered a core element of the pluripotent transcriptional network, but it is also specifically required for germ cell formation (Chambers et al. 2007). Thus, two possibilities could arise for explaining *Nanog* upregulation: that GS medium enhances ES cell pluripotency, or that culture supplementation with germline-related factors makes ES cells adopt an early germ cell pattern. This second possibility led us to investigate if germline pathways were involved in ES cell establishment when GS medium was used. PGC specification in the embryo is critically reliant on the signaling pathways elicited by bone morphogenetic proteins (BMPs), mainly by *Bmp4*, which activates *Alk2* (Hayashi et al. 2002). These signals direct a small group of proximal epiblast cells to enter the germ lineage and to express other germline markers as *Ifitm3* (*Fragilis), Dppa3 (Stella)* (Saitou et al. 2002) and *Ddx4* (Young et al. 2010). It has been suggested that during the ES cell derivation process, early germ cell specification-related genes are expressed under standard serum culture conditions (Tang et al. 2010). However, this germ cell-like state is facultative for the stabilization of pluripotency *in vitro,* since culture in 2i conditions enables the effective direct recruitment of ES cells skipping this germ cell-like state (Chu et al. 2011). In our study, germ cell specification-related genes were expressed in all culture media conditions and we could not observe a marked shift towards the germinal lineage with GS medium. The upregulation of *Stella,* which was observed in 2i conditions

could be associated to *Tet1* and *Tet2*-mediated demethylation (Ficz et al. 2011), since the 2i culture conditions have been reported to induce a global demethylation through Tet-driven hydroxylation (Leitch et al. 2013). Furthermore, the upregulation of *Stella* could be related with a different proportion of the subpopulations present in ESCs lines generated in 2i conditions (ICM-like and epiblast-like subpopulations) (Hayashi et al. 2008). The higher level of *Stella* and the lower level of other germ cell-related genes observed in 2i condition suggest that these efficient conditions for ESCs derivation could produce ESCs that are richer in the ICM-like subpopulation, meanwhile the ESCs derived in ES and GS conditions with lower levels of *Stella* and higher levels of germ cell specification-related genes could be rich in epiblast/germline-like subpopulations.

According to our observations, some imprinted genes could constitute ES cell quality indicators. The *Dlk1-Dio3* gene cluster consists of multiple imprinted genes, including *Mirg*, *Rian* and *Meg3* (Miyoshi et al. 2000). Recently, Stadtfeld *et al*. (Stadtfeld et al. 2010) reported that mouse induced pluripotent stem cells (iPSCs) with repressed expression of *Meg3*, contributed poorly to chimeras and failed to generate all-iPSC mice. In our study, we could observe that the highest *Meg3* expressions were triggered by the culture with GS medium, and interestingly, a positive correlation between *Meg3* expression and ES cell derivation efficiency was indeed demonstrated. *In vitro* culture can induce epigenetic instability in embryos and in ES cell lines derived from them, leading to biallelic expression of imprinted genes (Horii et al. 2010; Mann et al. 2004). Highest expression of imprinted genes *Rhox5* and *U2af1-rs1* was found in those groups with lowest ES cells derivation efficiencies, which could be caused by epigenetic instability or biallelic expression. Epigenetic instability in ES cells can also affect endogenous retrotransposable elements (REs). Accordingly, we observed upregulation of *IAP* in those groups with the lowest ES derivation efficiencies. Reactivation of *IAP* has been previously described in an ES cell line that lost the ability of germline transmission and started inducing epigenetic alterations in chimeric animals (Ramirez et al. 2006).

In summary, our observations suggest that the combined use of LIF during the *in vitro* embryo culture up to blastocyst stage and GS culture medium during the transition from blastocyst to ES cells synergically increase ES cell derivation efficiency. ESCs lines generated in these conditions accomplish pluripotency criteria and show an increased chimerism capacity. In addition, a correlation was observed between ES cell derivation efficiency and some imprinted genes expression.

Material and Methods

Embryo Collection

Mice were bred on a 14-h light/10-h dark cycle. Eight to ten week-old B6D2 F1 hybrid mice (C57Bl/6 x DBA) were superovulated by intraperitoneal injections of 7.5 IU of pregnant mare serum gonadotropin (PMSG) (Folligon; Intervet, Boxmeer, Holland) followed 48 h later by 7.5 IU of hCG (Chorulon; Intervet). On the same day of hCG injection (day 0), they were paired with male mice of the same strain. The mice were divided randomly into three groups: one *in vivo* study group (blastocysts collected at 3.5d) and two *in vitro* study groups for 2-cell embryo culture (collected at 1.5d) and 8-cell embryo culture (collected at 2.5d). Oviducts were excised by clamping the cornua, dissecting the peritoneum and fat between the ovary and tube and cutting the whole oviduct from the proximal end. After washing and flushing the oviduct from the proximal end with a 30-gauge needle, two-cell and eight-cell embryos were selected and collected by 100x stereoscopy.

In Vitro Embryo Culture

Two-cell and eight-cell embryos were placed in a droplet of KSOMaa or KSOMaa supplemented with 1000 U/ml LIF (KSOMaa-LIF) under oil in an environment of 5% CO_2 at 37°C. After 48 h (2-cell embryos) or 24 h (8-cell embryos), *in vitro* cultured blastocysts and *in vivo* blastocysts were plated individually in 96-well plates containing mitomycin-C (Sigma-Aldrich corporation St. Louis, MO, USA) treated mouse embryonic fibroblast (MEF) cells on 0.1% gelatin coated tissue plates in different media: (i) ES medium: Dulbecco's modified Eagle medium (DMEM plus 4500 mg/l glucose, glutaMAX, and pyruvate; Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS (PAA Laboratories Cölbe Germany), 2 mM glutamine, 1 mM MEM nonessential amino acids solution, 1 mM β-mercaptoethanol, 1000 U/ml LIF (ORF Genetics Iceland), and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin); (ii) GS medium: ES medium supplemented with 20 ng/ml Epidermal growth factor (EGF) (Peprotech, France), 10 ng/ml basic fibroblasts growth factor (bFGF) (Peprotech, France) and 10 ng/ml recombinant rat glial cell line-derived neurotrophic factor (GDNF) (Peprotech, France); and (iii) 2i medium: ES medium supplemented with 3 µM GSK3Beta inhibitor (Stemolecule^{TM} CHIR99021, Stemgent, San Diego, CA, USA) and 0.5 µM MEK inhibitor (Stemolecule^{TM} PD0325901, Stemgent, San Diego, CA, USA).

The blastocysts were allowed to attach to supportive MEFs, hatched and expanded. After 4d, outgrowths were recovered for gene expression analyses or trypsinized to derive ES cell lines by incubation in 0.05% Trypsin / 0.02% EDTA in Ca^{2+}-free and Mg^{2+}-free

Dulbecco's phosphate-buffered saline (PBS) at 37ºC and seeded directly into a well of a 96-well plate containing MEFs and ES, GS or 2i medium. Approximately 4 d after trypsinization, ESC colonies could be detected and expanded to a 24- or 96w depending on their confluency. Half of the cells were frozen when passage to a 35-mm dish was performed. For cell line expansion, cells were trypsinized every three days.

Differential ICM and TE Cell Counts

Blastocysts from the different groups were stained using a modification of the method originally described by Biggers et al. (Biggers et al. 2000). Blastocysts (3–5 at a time) were transferred from culture drops to acid Tyrode solution under constant observation for 5–15 s, until the zonae pellucidae were completely dissolved. They were next transferred to three rapid, successive washes in KSOMaa and then for 30 min into 10% rabbit antiserum to mouse red blood cells (Organon Teknika Corp., Durham, NC) in KSOMaa at 37ºC. After 30 min, embryos were transferred through three successive 5-min washes with KSOMaa and then into KSOMaa with 10% guinea pig complement (Gibco), 1 mg/ml bisbenzimide (Hoechst 33258), and 1 mg/ml propidium iodide (PI; Sigma) for 30 min at 37ºC. Each stained blastocyst was transferred to a clean glass slide, compressed under a glass coverslip, and visualized at 400x magnification in an inverted Nikon microscope with epifluorescence. Upon these treatments ICM cells were Hoechst positive, while PI marked TE cells. The numbers of nonmitotic, mitotic, and dead (i.e., degenerate and fragmented nuclei) cells were counted; being the total number of surviving cells the sum of nonmitotic and mitotic cells.

Analysis of marker gene expression by RT-PCR

The techniques for analysis of marker gene expression by RT-PCR have been described in detail previously (Bermejo-Alvarez et al. 2008) Total RNA was extracted using Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions. RT reaction was performed immediately according to the manufacturer's instructions (Gibco-BRL, Grand Island, NY, USA). 0.2 µM oligo (dT) and 0.5 µM random primers were added to RNA and heated 5 min, at 70ºC. RNA was reverse-transcribed at 42 °C for 60 min in a final volume of 40 µl containing 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNasin RNAse inhibitor (Promega, Madison, WI, USA), 10x MMLV-RT buffer with 8 mM DTT and 5 U MMLV high performance reverse transcriptase (Epicentre, Madison, WI, USA). Reaction was inactivated at 70ºC for 10 min.

The quantification of mRNA transcripts was performed by real-time quantitative (q)RT-PCR using a Rotorgene 6000 Real Time CyclerTM (Corbett Re- search, Sydney, Australia) and SYBR Green (Molecular Probes, Eugene, OR) as a double-stranded DNA-specific

fluorescent dye. PCR was performed by adding 2-µl of each sample to the PCR mix (Quantimix Easy Sig Kit, Biotools) containing the specific primers to amplify *Gapdh* as housekeeping; *Nanog*, *Pou5f1* (Oct3/4) and *Slc2a1* as pluripotency markers (Ramirez et al. 2007); *Bmp4*, *Alk2*, *Ifitm3, Dppa3* and *Ddx4* as germ line differentiation markers (Cyril Ramathal 2011) and *Meg3*, *Rhox5*, *IAP* and *U2af1-rs1* as imprinted genes. Primer sequences and the approximate sizes of the amplified transcripts are given in Supplementary Table 1. Details of the qRT-PCR procedure have been described elsewhere (Ramirez et al. 2006). The PCR protocol included an initial step of 94°C (2 min), followed by 35 cycles of 94°C (15 s), 56°C (30 s) and 72°C (30 s). Fluorescent data acquisition temperature for each primer is detailed in Supplementary Table 1. The melting protocol consisted of 60 s at 40°C for and then heating from 50 to 94°C, holding at each temperature for 5 s while monitoring fluorescence. For qRT-PCR, three groups of cDNA per experimental group were used in two repetitions for all genes of interest.

Table 2: Primer sequences, annealing temperatures, and the approximate sizes of the amplified fragments of all transcripts.

Gene	Primer Sequences 5'-3'	Size	GeneBank Accesion No.	Acquisition Temperature (ºC)
Gapdh	ACCCAGAAGACTGTGGATGG ATGCCTGCTTCACCACCTTC	247	BC102589	86
Nanog	AGGGTCTGCTACTGAGATGCTCTG CAACCACTGGTTTTTCTGCCACCG	363	NM_028016	86
Pou5f1	GGAGAGGTGAAACCGTCCCTAGG AGAGGAGGTTCCCTCTGAGTTGC	312	NM_013633	87
Slc2a1	CCAGCTGGGAATCGTCGTT CAAGTCTGCATTGCCCATGAT	688	NM_011400.3	86
Ifitm3	TGCCTTTGCTCCGCACCAT GGGTGAAGCACTTCAGGACC	531	NM_025378.2	84
Alk2	GGAGTAATGATCCTTCCTGTGC TCTTACACGTCATCTTCCCCTG	216	NM_007394.3	84
Stella	GCAATCTTGTTCCGAGCTAG CTGGATCGTTGTGCATCCTA	312	AY082485.1	83
Ddx4	GCTCAAACAGGGTCTGGGAAG GGTTGATCAGTTCTCGAG	564	JN951543.1	83
Bmp4	TGTGAGGAGTTTCCATCACG TTATTCTTCTTCCTGGACCG	547	BC013459.1	86
Meg3	TCTGCCTGGCTTTCGCCCCA AAGCACCATGAGCCACTAGG	932	NR_003633.3	86
Rhox5	AGAGATGAGCAAGGTGCACA CGAACCTAGAGCCCTGGAG	237	NM_008818.2	86
U2af1-rs1	CGTGGGAAAGGTGATTCAGT GCTGCCTTAGCTGGGCTCAGG	566	NM_011663.3	84
IAP	GGGTATTGTTGAGCGTGCGC TCGGGTGAGTCTTTCTGGTAC	333	XM_001477167.1	82

The comparative CT method was used to quantify expression levels (Miranda et al. 2011). Quantification was normalized to the endogenous control *Gapdh*. Fluorescence was acquired in each cycle to determine the threshold cycle in the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. According to the comparative CT method, the ΔCT value was determined by subtracting the *Gapdh* CT value for each sample from each gene CT value. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2-ΔΔCT. For those genes specifically expressed in the ICM, a second normalization of the expression levels was performed taking into account the ratio ICM/Total cells. Data on mRNA expression were analyzed using Prism 5 (Graph pad software, CA, USA) software package. Differences in mRNA expression were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) package by one-way repeated-measures ANOVA with arcsine data transformation, and significance determined using Holm-Sidak post hoc test. A *P* value ≤ 0.05 was considered statistically significant.

Karyotype analysis.

To prepare chromosome spreads of the ES cell lines, cells were arrested in metaphase by supplementing the culture medium with 0.1 μg/ml colcemid for 4 h at 37°C in a 5% CO_2 air atmosphere. Cells were then treated with trypsin-EDTA for 2 min at 37°C and after pipetting, the single cell suspension was washed twice with PBS by centrifugation at 200 G for 5 min. The resulting pellet was exposed to a hypotonic shock by resuspension in 0.075 M KCl for 15 min at 37°C. After a second centrifugation step the hypotonic solution was removed and the pellet fixed with a methanol/acetic acid solution (3:1; vol/vol) by gently pipetting. Ten min later, cells were pelleted and fixed again. Before slide mounting, cells were washed twice with PBS and dropped into slides, which were dried overnight at 55°C, stained with freshly made 10% Giemsa solution for 30 min and rinsed with distilled water. Finally, chromosome number was counted in at least 30 metaphase cells for each cell preparation.

Chimera formation

For chimera formation, we used blastocyst ES cell injection procedure (Ramírez et al. 2009). Ten cells were injected into the blastocoel of 3.5 dpc blastocysts of CD1 mice using Eppendorf micromanipulators (Eppendorf TransferMan NK 2, Hamburg, Germany). The blastocysts cultured overnight in KSOM, were returned to the oviducts of 0.5 dpc pseudopregnant CD1 foster mothers on the next day of microinjection. To generate chimeric mice, the embryos were allowed to develop to term.

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***Dazl*-GFP mice model generated by a two-step ESC-based strategy to identify pluripotent and germ cells**

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Chapter III / Capítulo III

Summary

Numerous germ cell and premeiotic markers have been found to be expressed in several pluripotent cell types, which may indicate a common germ cell origin for them.

Deleted in azoospermia like (*Dazl*) is a gene expressed in germ cells through different developmental stages, whose ablation results in infertility in both sexes. However, recent studies suggest that *Dazl* could have another role related to pluripotency. The aim of this experiment was to track *Dazl* expression in different tissues along development, and to investigate the existence of pluripotent cells from the fertilized embryo up to adulthood. For this purpose we generated a *Dazl*-eGFP-transgenic mouse and assayed the ability of *Dazl*-driven-eGFP to analyze preimplantational embryo development, foetal, neonatal and adult tissues, and *in vitro* differentiation from embryonic stem cells (ESCs) to embrioid bodies (EBs) and to primordial germ cells (PGCs).

During preimplantational embryo stages, eGFP was detected from zygote to blastocyst. Later, eGFP was expressed in genital ridges and in neonatal gonads of both sexes. In adult mice, *Dazl*-eGFP expression was found during spermatogenesis from spermatogonia to elongating spermatid and in the cytoplasm of oocytes along follicular development. eGFP mRNA was detected also in intestine and bone marrow. Fluorescence was maintained along *in vitro Dazl*-eGFP ESCs culture and differentiation to EBs and to PGCs, indicating that *Dazl* marks both pluripotent and germ cells *in vitro*.

In conclusion, besides its largely known function in germ cell development, *Dazl*-eGFP expression in other tissues harboring pluripotent cells, as well as during embryo development and *in vitro* ESCs differentiation, confirms *Dazl* role in pluripotency. Therefore, *Dazl*-eGFP transgene could be used to explore the presence of pluripotent cells in different tissues.

Resumen

Numerosos marcadores de linaje germinal y premeióticos se expresan también en varios tipos de células pluripotentes, lo que podría indicar un origen germinal común para todos ellos.

Deleted in azoospermia like (*Dazl*) es un gen que se expresa en células germinales a lo largo de diferentes estadios del desarrollo, y cuya ausencia produce infertilidad en ambos sexos. El objetivo de este experimento fue hacer un seguimiento de la expresión de *Dazl* en diferentes tejidos a lo largo del desarrollo, e investigar la presencia de células pluripotentes desde el embrión fertilizado hasta la edad adulta. Para esto se generó un ratón transgénico *Dazl*-eGFP y se analizó el desarrollo del embrión preimplantacional y su expresión en tejidos fetales, neonatales y adultos, además de la diferenciación *in vitro* desde células troncales embrionarias (ESCs) hacia cuerpos embrioides (EBs) y hacia células primordiales germinales (PGCs).

Durante los estadios embrionarios preimplantacional, eGFP fue detectado desde el cigoto hasta el blastocisto. Posteriormente, se observó expresión de eGFP en anillos germinales y en gónadas neonatales de ambos sexos. En el ratón adulto, *Dazl*-eGFP se expresó durante la espermatogénesis desde el estadio de espermatogonia hasta el de espermátida elongada, y durante el desarrollo folicular en el citoplasma de los ovocitos. Además se detectó ARNm de eGFP en el intestino y en la médula ósea. La fluorescencia se mantuvo a lo largo del cultivo y la diferenciación *in vitro* de las ESCs *Dazl*-eGFP hacia EBs y PGCs, indicando *Dazl* se expresa tanto en células pluripotentes como en células germinales *in vitro*.

En conclusión, aparte de su conocida función durante el desarrollo de las células germinales, la expresión de *Dazl*-eGFP en otros tejidos en los que existen células pluripotentes, así como durante el desarrollo embrionario y en la diferenciación *in vitro* de ESCs, confirman que *Dazl* tiene un rol relacionado con la pluripotencia. Por lo tanto, el transgen *Dazl*-eGFP podría ser usado para analizar la presencia de células pluripotentes en diferentes tejidos.

Dazl-GFP mice model generated by a two-step ESC-based strategy to identify pluripotent and germ cells

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Abstract

Background: Deleted in azoospermia like (*Dazl*) gene is preferentially expressed in germ cells; however, recent studies indicate that it may have pluripotency-related functions.

Methods: We have generated *Dazl*-GFP-transgenic mice and assayed the ability of *Dazl*-driven-GFP to mark preimplantation embryo development, foetal, neonatal and adult tissues, and *in vitro* differentiation from embryonic stem cells (ESCs) to embryoid bodies (EBs) and to primordial germ cells (PGCs). *Dazl*-GFP mice were generated by a two-step ESC-based strategy, which enabled primary and secondary screening of stably transfected clones before embryo injection, obtaining *Dazl* reporter mice with physiologically appropriate regulation of GFP expression.

Results: During preimplantational embryo stages, GFP was detected from zygote to blastocyst. At e12.5, GFP was expressed in gonadal ridges and in neonatal gonads of both sexes. In adult mice, *Dazl*- driven GFP expression was found during spermatogenesis from spermatogonia to elongating spermatid and in the cytoplasm of oocytes along follicular development. However, GFP mRNA was also detected in other tissues harboring multipotent cells as intestine and bone marrow. Fluorescence was maintained along *in vitro Dazl*-GFP ESCs differentiation to EBs in ESC medium without LIF, and in PGCs.

Conclusions: Besides its largely known function in germ cell development, *Dazl* has an additional role in pluripotency, supporting these transgenic mice as a valuable tool for the prospective identification of stem cells from several tissues.

Background

Deleted in azoospermia like (*Dazl*) gene is expressed in germ cells through different developmental stages. Several studies based on knock-out mouse models have concluded that *Dazl* ablation results in infertility in both male and female mice, which has been explained mainly by a meiotic failure [1-4]. Thus, in males, *Dazl* deficiency results in severe disruption of testicular histology, including an almost complete absence of germ cells due to a failure of most A_{al} spermatogonia to differentiate into A_1 spermatogonia, and to an arrest in the pachytene stage of the meiotic prophase [1]. In females, tiny ovaries with marked deficiency of oocytes can be detected as early as e19 female fetus, which is also explained by a failure to complete meiotic prophase [2, 3].

However, the fact that *Dazl* is not only expressed in premeiotic cells suggests that it also exerts other functions different to meiosis regulation. To assess putative specific roles of *Dazl* in processes such as germ cell differentiation, it is crucial to determine its expression pattern in diverse cell types at different stages of development. In the germline, *Dazl* expression begins as early as e11.5 in primordial germ cells (PGCs) located in gonadal ridges [5, 6], whereas in adult mice, *Dazl* is expressed in B-spermatogonia, preleptotene, zygotene and pachytene spermatocytes in males; and in the cytoplasm of oocytes and in follicular cells in maturing follicles in females [2]. However, recent studies have linked *Dazl* expression with pluripotent cells out of the germ cell lineage such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), embryonal carcinoma cells (ECCs) [7], embryoid bodies (EBs) [8, 9], mouse bone marrow mesenchymal stem cells [10, 11] and human amniotic fluid cells [12], suggesting a possible broader role of *Dazl* in pluripotency.

Transgenic mice where a reporter gene expression is driven by a known gene promoter constitute an ideal model to study gene localization in several tissues along different developmental periods, as not only allows easy identification of cells expressing the transgene, but also the reporter gene does not interact with biological processes. In this study we have developed a transgenic mouse expressing GFP under *Dazl* promoter to determine the expression pattern of *Dazl* during mouse development *in vivo* and in differentiation studies *in vitro*, and we have found that *Dazl* marks not only germ cells, but it is also expressed in pluripotent cells.

Methods

Culture and transformation of ESCs

Using previously described methods, a XY ESC line was derived in our laboratory from fertilized embryos in hybrid female mice (C57BL/6 x DBA/2) [13]. The ESCs line was initially checked for pluripotency based on the expression of molecular markers specific for ESCs [14] and for its ability to successfully produce chimeras showing germline transmission. Karyotype analysis was performed as previously described [15].

ESCs were maintained in mitomycin-C treated (Sigma-Aldrich corporation, St. Louis, MO, USA) mouse embryonic fibroblast (MEF) cells on 0.1% gelatin-coated tissue plates and cultured in ESC medium composed of Dulbecco's modified Eagle medium (DMEM plus 4500 mg/l glucose, glutaMAX, and pyruvate; Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS (PAA Laboratories, Cölbe, Germany), 2 mM glutamine, 1 mM MEM nonessential amino acids solution, 1 mM β-mercaptoethanol, 1000 U/ml leukemia inhibitory factor (LIF), and an antibiotic mixture containing 100 U/ml penicillin and 100 mg/ml streptomycin.

ESCs were transfected with the linearized *Dazl*-GFP transgene [16] by electroporation as follows: 10 µg of the linearized *Dazl*-GFP construction were electroporated into $3x10^6$ cells using a Multiporator (Eppendorf, Hamburg, Germany) and a pulse of 300 V for 500 µs in a 4 mm cuvette. Cells were allowed to recover for 24 h before Geneticin 418 (G418) was added to a final concentration of 150 µg/ml. Cellular clones were selected by geneticin resistance over 7 days and screened for GFP expression under fluorescence microscopy. DNA from the transformed ESCs was processed for PCR analysis to confirm transgene integration using standard protocols [15].

Production of Dazl-GFP transgenic mice

For *Dazl*-GFP transgenic mice production, eight-cell stage embryos from CD1 mice were used for ESCs microinjection using Eppendorf micromanipulation equipment with vario cell injectors to produce healthy, full germline-competent mice from hybrid ESCs [17]. Mice were kept on a 14-h light / 10-h dark cycle. CD1 female mice (8–10 wk old) were superovulated by intraperitoneal injection of 7.5 IU of equine chorionic gonadotropin (eCG; Intervet, Boxmeer, Holland), followed 48 h later by 7.5 IU of human Chorionic Gonadotropin (hCG; Lepori, Farma-Lepori, Barcelona, Spain). On the day of hCG injection, female mice were paired with male mice from the same strain to allow mating. Eight-cell embryos were collected 2.5 days post coitum (dpc) as previously described [18].

A single ESC was injected into eight-cell stage CD1 mouse embryos using Eppendorf micromanipulators (Eppendorf TransferMan NK 2, Hamburg, Germany). Embryos were cultured for 24 h in potassium simplex optimization medium (KSOM) supplemented with

aminoacids and 4% ESC medium in droplets overlaid with mineral oil in a humidified atmosphere of 5% CO_2 at 37°C. After culture, microinjected blastocysts and compacted morulae were transferred into the left uterus of a 2.5 dpc pseudopregnant CD1 female [17]. DNA from newborn mice was then processed for PCR analysis to identify GFP integration using standard protocols.

All the animals were kept in an animal house and handled using procedures and protocols approved by the Animal Care and Ethics Committee (Informe CEEA 2009/009) of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA, Madrid) and conducted in accordance with the European Union Directive and Spanish legislation.

RNA isolation, cDNA synthesis and PCR

Total RNA was extracted from testis, ovary, brain, kidney, small intestine, large intestine, bone marrow, liver, skin, heart and lung of transgenic mice at different ages (e16.5 fetus, newborn and adult), and from EBs using the Ultraspect™ RNA Isolation System following the manufacturer´s instructions (Biotecx Lab. Inc., Houston, Texas, USA) [19]. Three samples of each group were processed. RNA was extracted and then dissolved in nuclease-free water (Promega Corporation, Madison, WI, USA) and treated with 2U of RQ DNAse I (Promega Corporation, Madison, WI, USA) at 37 °C for 30 min to ensure that the only source of DNA in the polymerase chain reaction (PCR) was cDNA from cellular RNA. Immediately after extraction, the retrotranscription (RT) reaction was carried out following the manufacturer's instructions to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure, allowing Random Primer and Oligo dT annealing, and the RT mix was then completed with the addition of 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNAsin RNAse inhibitor (Promega, Madison, WI, USA), MMLV HP RT 10X reaction buffer, 5 mM DTT and 50 U MMLV high performance reverse transcriptase (Epicentre, Madison, WI, USA). Tubes were first incubated at room temperature for 10 min and then at 42°C for 60 min to allow the reverse transcription of RNA, followed by 70°C for 10 min to denature the RT enzyme [20].

Qualitative analysis of mRNA transcripts was performed by PCR, by adding a 2-µL aliquot of each sample to the PCR mix containing the specific primers to amplify glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*: 5´-AGGTCGGTGTGAACGGATTTG / TGTAGACCATGTAGTTGAGGTCA) and GFP (5´-TGAACGGCATCGAGCTGAAGG / TCCAGCAGGACCATGTGATCGC). PCR conditions were 94 °C for 3 min followed by 40 cycles (94 °C 15 s, 56 °C 20 s, 72 °C 30 s) and 72 ºC for 5 min.

The quantification of mRNA transcripts was carried out by real-time quantitative PCR, contrasting relative levels of GFP and glyceraldehyde-3-phosphate dehydrogenase

(*Gapdh*) as housekeeping, in two repetitions per sample. PCR was performed by adding a 2-µL aliquot of each sample to the PCR mix containing the specific primers to amplify target genes. PCR conditions were tested to achieve efficiencies close to 1, which were 94 °C for 3 min followed by 40 cycles (94 °C 10 s, 56 °C 30 s, 72 °C 10 s, and 10 s of fluorescence acquisition – SYBR -) (Rotor Gene 2000, Corbett Research), and then the comparative cycle threshold (C_T) method was used to quantify expression levels. Quantification was normalized to the endogenous control, *Gapdh*. To avoid primer dimers artifacts, fluorescence was acquired in each cycle at a temperature higher than the melting temperature of primer dimers (specific for each product). Then, the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background was determined for each sample. According to the comparative C_T method, the ΔC_T value was determined by subtracting the endogenous control (*Gapdh*) C_T value for each sample from GFP gene C_T value of the sample. Calculation of $\Delta\Delta C_T$ involved using the highest sample ΔC_T value (i.e., the sample with the lowest target expression) as a constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta C}{}_T$ [21].

Immunohistochemistry

Tissues were fixed in Bouin's fluid for 6 h, this fluid replaced with 70% ethanol and the tissue then paraffin embedded and sectioned (5 µm). Slides were deparaffinated by incubating four times for 4 min in xylene, and rehydrated (100, 96, 70% ethanol, water; 4 min each). Antigens were retrieved by heating the slides immersed in trisodic citrate buffer 10 mM for 3 min in a pressure cooker. Next, endogen peroxidase was blocked in 0.3% hydrogen peroxide (Sigma-Aldrich, Germany) for 30 min at room temperature, and the tissue sections permeabilized with PBS-0.1% Tween ® 20 (MERCK-Schuchardt, Germany) for 5 min, blocked in 10% normal goat serum (Vector, Burlingame, CA, USA) for 30 min and treated with the Avidin/Biotin blocking kit (Vector, Burlingame, CA, USA). The slides were then incubated overnight at 4ºC with rabbit anti-GFP antibody diluted 1:500 (Genetex, San Antonio, TX, USA). This was followed by incubation for: 1 h at room temperature with a 1:300 dilution of biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector, Burlingame, CA, USA); 30 min at room temperature with the Vectastain Elite ABC Kit (Vector, Burlingame, CA, USA); and 10 min at room temperature with the Vector NovaRED substrate Kit (Vector, Burlingame, CA, USA). Coverslips were mounted using Vectamount mounting medium (Vector, Burlingame, CA, USA) and observed by brightfield microscopy (Nikon OPTISHOT-2).

Immunofluorescence

To localize *Dazl*-GFP expression, blastocysts were washed in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and fixed in 4% paraformaldehyde (Panreac, Barcelona, Spain) for 10 min at room temperature. Then, they were permeabilized by incubation in PBS with 5% normal goat serum (Vector, Burlingame, CA, USA) and 1% Triton X-100 (Sigma, Steinheim, Germany) for 45 min at room temperature. After permeabilization, blastocysts were incubated overnight at 4 ºC in PBS containing 1% BSA, 1% normal goat serum and 1:500 rabbit monoclonal anti-GFP antibody (E385, Abcam, England). Following incubation, blastocysts were washed twice in PBS - 1% BSA and incubated in PBS supplemented with 1%BSA, 1% normal goat serum, 1:300 goat polyclonal Secondary Antibody (Alexa Fluor® 488, Abcam, England) and 0.01 mg/ml DAPI (Sigma, Steinheim, Germany) for 2 h at room temperature [22]. Finally, embryos were washed three times in PBS - 1% BSA, mounted in microdrops with Fluoromount G (EMS, Hatfield, UK), and examined by confocal microscopy. Negative controls were performed in the same way using wild type blastocysts, and with omission of the primary antibody before secondary antibody addition.

Isolation and culture of Dazl-GFP primordial germ cells (PGCs)

C57/DBA2 female mice were mated with *Dazl*-GFP males, and 12.5 dpc embryos were recovered and dissected. Gonadal ridges expressing the transgene were collected under fluorescence stereoscopy and were dissociated into single cells by incubation at 37 ºC in 0.05% Trypsin/0,02% EDTA in Ca^{2+}-free and Mg^{2+}-free Dulbecco's phosphate-buffered saline for 10 min with gentle pipetting. Cells were seeded into a well containing inactivated MEF in ES medium supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) and 50 ng/ml stem cell factor (SCF) [23, 24]. The medium was replaced every 24-48 hours and cells were dissociated by 0.05% Trypsin / 0.02% EDTA incubation and replated on a fresh MEF feeder layer when macroscopic colonies appeared.

Dazl-GFP ESCs differentiation

ES cells were differentiated to embryoid bodies (EBs) following standard methods [15]. Briefly, ESCs were trypsinized and back-plated for 15 minutes to deplete fibroblasts and then placed in non-adherent 10 cm bacterial-grade Petri dishes ($5x10^5$ cells per dish) in ESC medium without LIF supplementation.

EBs differentiation towards primordial germ cells (PGCs) was performed at day 7 of EBs formation. Medium with floating EBs was centrifuged at 290g for 5 min and the resulting pellet was incubated first at 37 ºC in collagenase (1mg/ml) during 30 min, and then in 0.05% Trypsin / 0,02% EDTA in Ca^{2+}-free and Mg^{2+}-free Dulbecco's phosphate-buffered

saline at 37 ºC during 20 min. The reaction was blocked with ESC medium, and single cells were cultured onto inactivated MEFs for 8 days in the presence of 2 µM Retinoic acid [25].

Results

Production of Dazl-GFP transgenic mice

The promoter activity of a 1.7 Kb sequence located upstream of the mouse *Dazl* translation start site has been described as sufficient to drive robust germ cell-specific expression in transgenic mice produced by pronuclear microinjection but failed to show strong reporter gene expression in embryonic or adult tissues [16]. To ensure strong transgene expression,we used an alternative method to generate transgenic mice with the goal to screen for optimal level of GFP expression before the production of live animals. Two *Dazl*-GFP transfected ESC lines were selected for high GFP expression and were injected into 60 eight-cell stage embryos of CD1 mice. Fifty-nine of them developed to blastocysts and were transferred to the uterus of 3.5 dpc pseudopregnant CD1 female. Pregnancies were allowed to develop to term and 18 newborn mice were obtained (10 and 8 animals for each line). The efficiency of the generation of F0 ESC mice was quantified by the percentage of ESC coat contribution and germline transmission. Two chimeric mice with more than 75% ES cell coat color and germline transmission were produced per line. Similar expression pattern was found between both transgenic lines (not shown); thus, all the experiments were performed with one of them.

Transgene Expression in vivo

Preimplantation embryos were observed by fluorescence microscopy and a cytoplasmic localization of the transgene was evident. Fluorescence could be detected from zygote to blastocyst stage, although it was weaker in the eight-cell stage (Fig 1a). *Dazl*-GFP expression in expanded blastocysts was analyzed by immunofluorescence and it was detected in the cytoplasm of both the inner cell mass and the trophectoderm cells with a granular pattern in some areas (Fig 1d,e).

Dazl-GFP expression could be detected in male and female fetal gonads by fluorescence stereoscopy as early as e12.5 (Fig 1b,c), resembling endogenous *Dazl* expression pattern [5]. Newborn and adult testis also showed strong transgene expression (Fig 3 d,e); in contrast, fluorescence could not be detected above background in the ovary.

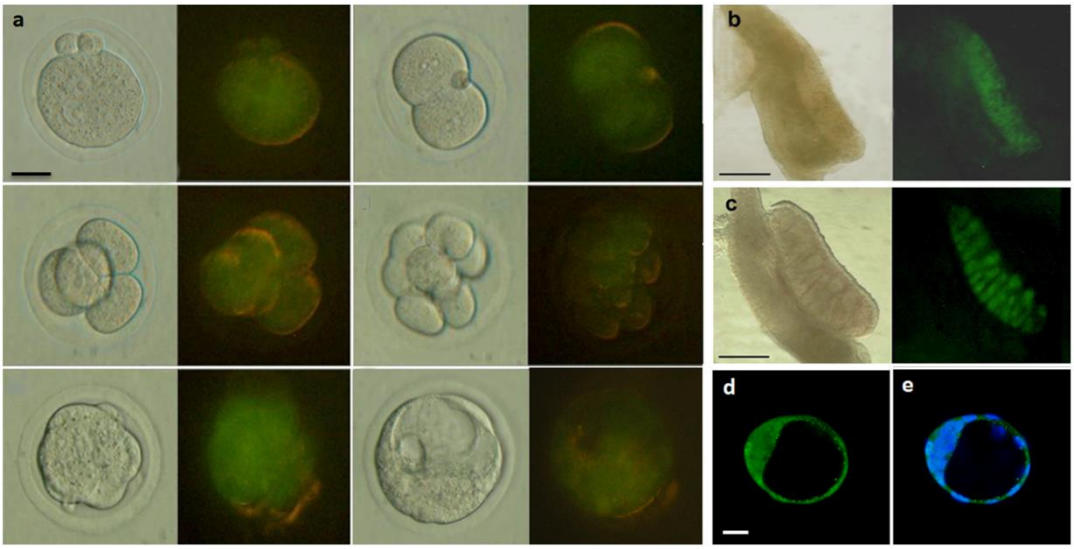

Figure 1. ***Dazl*-GFP transgene expression in preimplantation embryos and gonadal ridges.** (a) Brightfield and fluorescence micrographs of zygote, 2-cells embryo, 4-cells embryo, 8-cells embryo, morula and expanded blastocyst. Bar = 20 µm. (b) Brightfield and fluorescence micrographs of e12.5 female and (c) male gonadal rings. Bars = 500 µm. Immunofluorescence confocal sections of (d) *Dazl*-GFP and (e) DAPI and *Dazl*-GFP merged channels of a blastocyst. Bar = 15 µm

Transgene expression was next analyzed by qualitative PCR analysis performed in a panel of tissues: testis, ovary, brain, kidney, small intestine, liver, skin, heart and lung, isolated from fetus (e16.5), newborn and adult mice; and large intestine and bone marrow isolated from newborn and adult *Dazl*-GFP mice. GFP transcripts were detected in testis and ovary at all stages, in intestine in fetus, and in bone marrow in newborn and adult. Subsequently, we quantified GFP mRNA in those tissues expressing the transgene by real-time qRT-PCR (Fig 2). Highest expression was found in testis, being much higher in adult than in fetus and newborn. GFP transcript expression in ovaries was higher in fetus and newborn, but very low in adult. Considerable expression was found in newborn large intestine, as well as in fetal and newborn small intestine and in newborn and adult bone marrow.

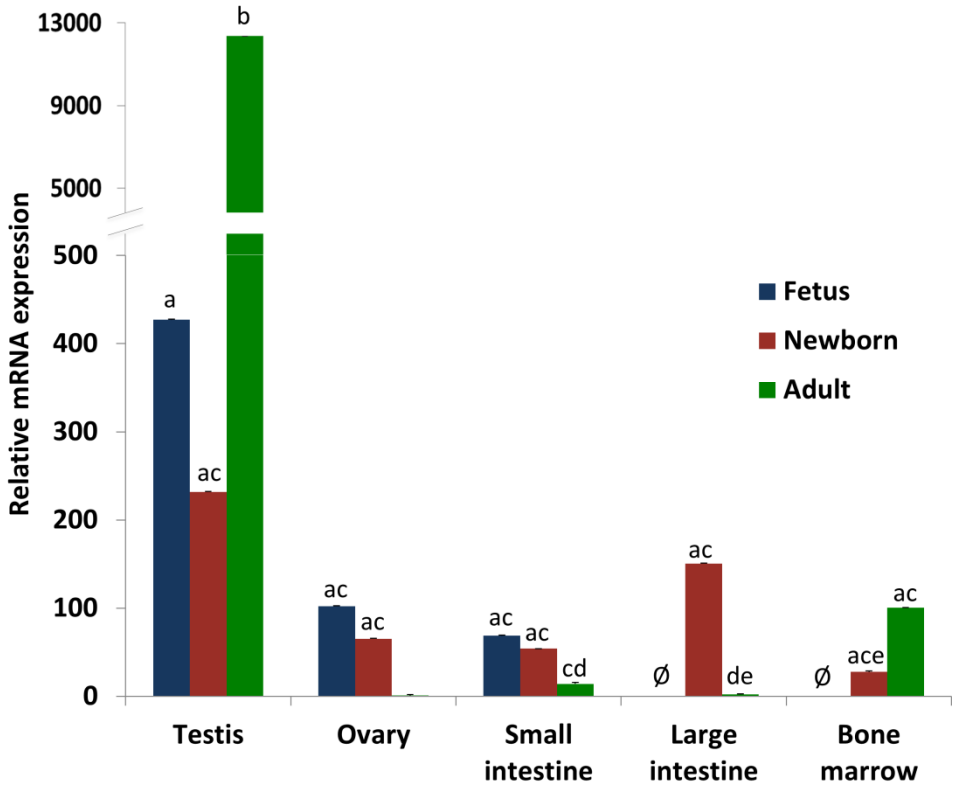

Figure 2. Relative GFP mRNA expression in tissues from e16.5 fetus, newborn and adult transgenic *Dazl*-GFP mice. Different letters indicate statistical differences in gene transcription at P<0.05; 1-way ANOVA, Holm-Sidak post-hoc test.

Subsequently, GFP protein location was analyzed by immunostaining in sections from transgenic mice tissues. All seminiferous tubules in adult testis stained positive for GFP (Fig 3a,b,c). *Dazl*-GFP expression was restricted to germ cells, being absent in interstitial Leydig cells and in Sertoli cells lining the basement membrane of the seminiferous tubules. Initial expression was found in spermatogonia, followed by strongest expression in spermatocyte and also high expression in round and elongating spermatid, although spermatozoa were not stained (Fig 3 f). Strong GFP expression was also observed in some small round cells located near the basement membrane in certain seminiferous tubules. Although their distribution pattern was similar to Spermatogonial Stem Cells (SSC), further analysis would be necessary for a better characterization (Fig 3g). In fetal sections, GFP staining was restricted to primordial germ cells in the gonadal ridges from male and female as early as e12.5 (Fig 3h). Expression was also restricted to gonocytes in e15.5 fetal testis (Fig 3i).

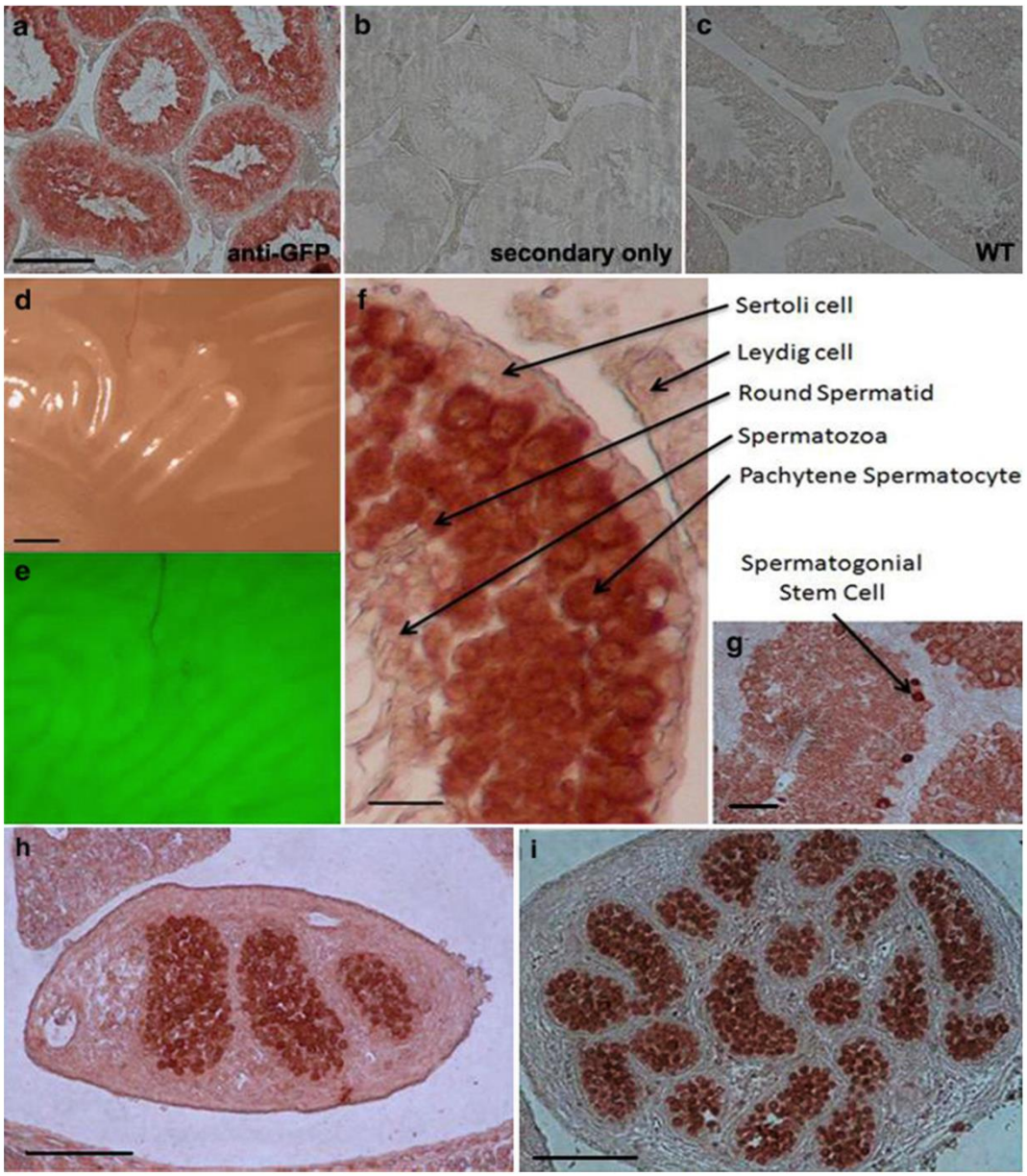

Figure 3. **Male germ cell-specific *Dazl*-GFP expression *in vivo*.** (a) Immunohistochemistry (IHC) staining of adult testis sections from transgenic *Dazl*-GFP mouse with anti-GFP antibody, (b) control with secondary antibody only, and (c) control of adult testis section from WT mouse with anti-GFP antibody. Bar = 100 µ. (d) Brightfield and (e) fluorescence micrographs of a testicle from an adult *Dazl*-GFP transgenic mouse. Bar = 400 µm. (f) IHC staining of GFP in a seminiferous tubule section from an adult transgenic *Dazl*-GFP mouse. Bar = 20 µm. (g) Seminiferous tubule section showing strongly stained spermatogonial stem cells. Bar = 50 µm. (h) IHC anti-GFP staining of an e12.5 gonadal ridge section and (i) e15.5 testis section from transgenic *Dazl*-GFP mouse. Bar = 200 µm.

In adult (Fig 4a,b) and newborn (Fig 4c) ovary sections, *Dazl*-GFP was detected in germ cells but not in somatic cells. Most intense signal was observed in the cytoplasm of oocytes of primordial follicles. Signal persisted in oocytes of primary, secondary and antral follicles and also appeared in the antrum of antral follicles. In contrast, staining was not detected in granulosa and thecal cells, or in *corpora lutea*.

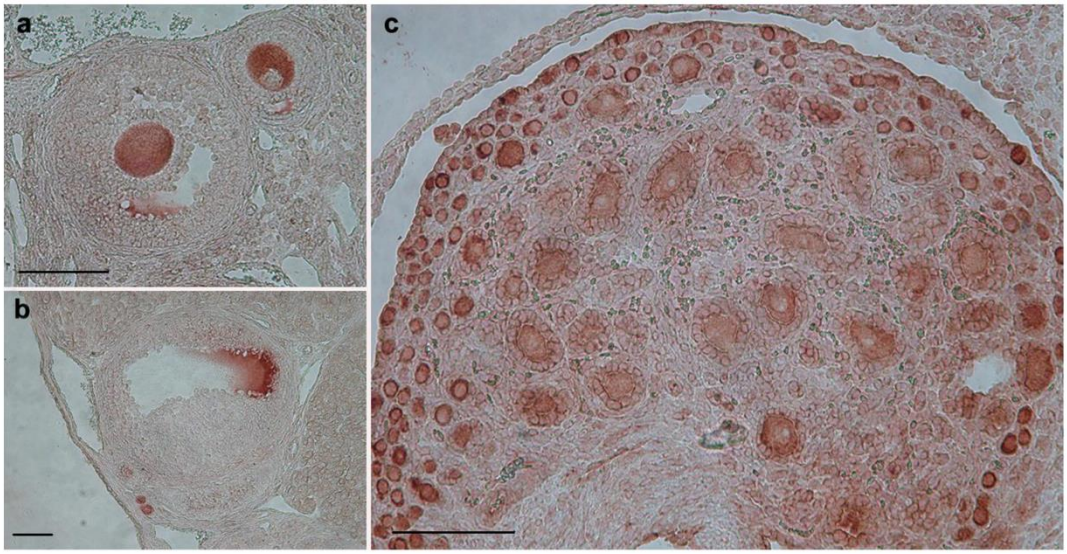

Figure 4. Female germ cell-specific *Dazl*-GFP expression *in vivo*. Immunohistochemistry anti-GFP staining of adult ovary sections from transgenic *Dazl*-GFP mouse showing (a) secondary and antral follicles, and (b) preovulatory and primordial follicles. Bars = 100 µm. (c) IHC anti-GFP staining of a neonatal ovary section from transgenic *Dazl*-GFP mouse. Bar = 50 µm.

Transgene expression in vitro

Fluorescence microscopy allowed us to isolate e12.5 male and female GFP-positive gonadal ridges (Fig 1b,c), which were processed separately to establish male and female GFP-positive Primordial Germ Cells (PGCs) primary cultures. PGCs colonies appeared at day 6 of culture, showing a flattened morphology, with strong and uniform Alkaline Phosphatase (AP) staining (Fig 5a). Transgene expression could still be observed by fluorescence microscopy after 1 month and three cell passages from isolation (Fig 5b).

Dazl-GFP ESCs presented strong fluorescence, which was also maintained in culture along passages. When ESCs were differentiated towards embryoid bodies (EBs) in ESC medium without LIF for up to 19 days, fluorescence was retained along differentiation (Fig 5c). Then, EBs were differentiated towards the germinal lineage by disaggregation and culture onto mouse embryonic fibroblasts with retinoic acid supplementation, which promotes rapid ESCs differentiation into PGCs [25]. Two different types of colonies appeared that

showed *Dazl*-GFP expression by fluorescence microscopy: ESCs, and flattened and densely packed AP-positive colonies resembling PGCs (Fig 5 d), which maintained *Dazl*-GFP expression in culture (Fig 5e,f).

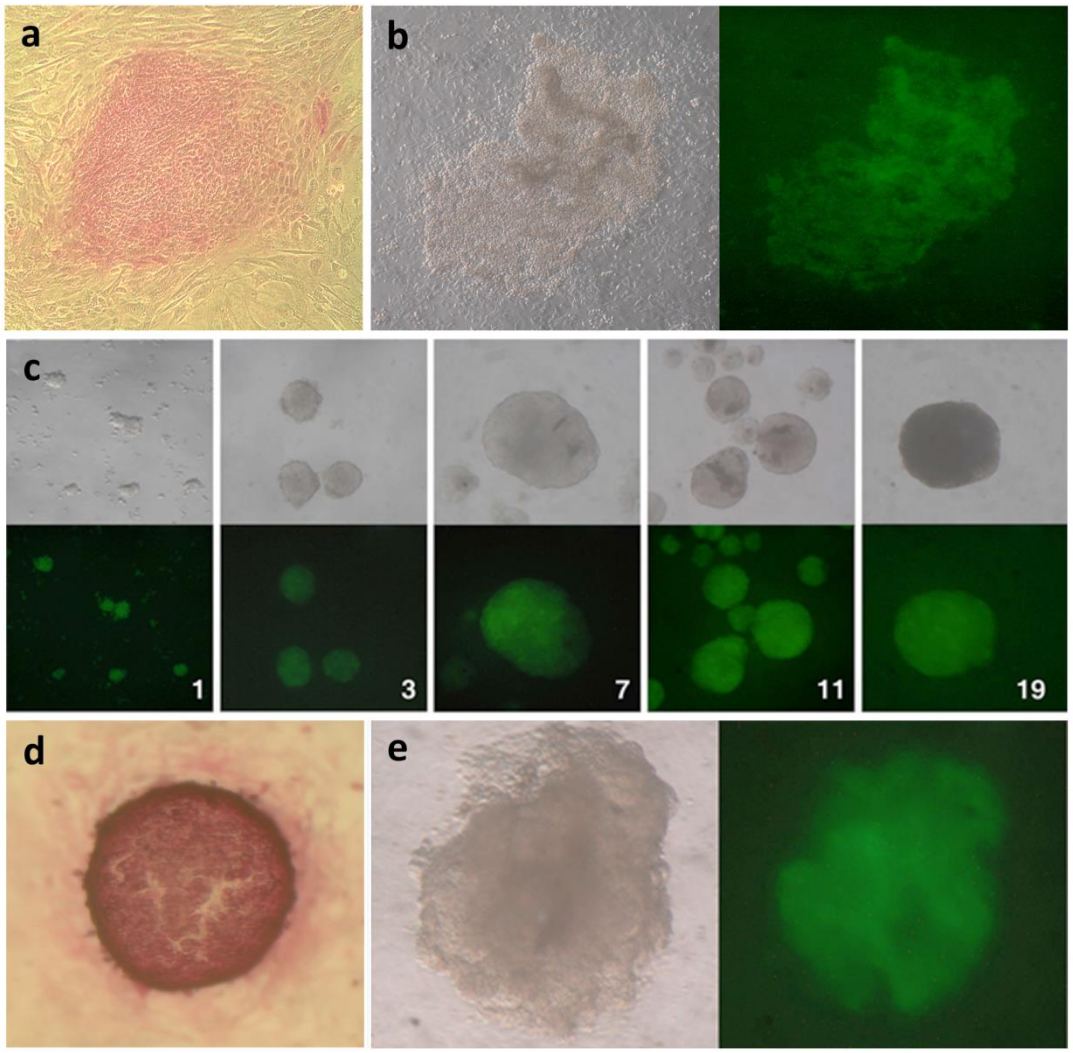

Figure 5. *Dazl*-GFP expression *in vitro*. (a) PGCs colony obtained from gonadal ridges showing Alkaline Phosphatase staining. (b) Brightfield and fluorescence micrographs of a PGC colony derived from gonadal ridges. (c) *Dazl*-GFP expression during ESCs differentiation: brightfield and fluorescence micrographs of EBs at days 1, 3, 7, 11 and 19 of differentiation. (d) Alkaline Phosphatase staining and (e) brightfield and fluorescence micrographs of PGCs derived from EBs.

Discussion

The *Dazl*-GFP transgenic mouse generated herein by injection of highly expressing transgenic ESCs into eight-cell embryos constitutes a powerful and reliable tool to determine the expression of *Dazl* along development. To ensure transgene expression in pluripotent cells, we used a two-step ES cell-based visual screening method, and we produced two transgenic lines. GFP was expressed *in vivo* in the germline from PGCs through maturation into gametes, and in non-gonadal adult tissues harboring multipotent stem cells, as well as during preimplantation embryo development. These transgenic lines are a good model to identify and enrich for putative stem cell populations and to isolate germ cell populations derived from ESCs.

GFP protein expression was observed by fluorescence as early as e12.5 in both male and female gonadal ridges restricted to primordial germ cells, in agreement with previous studies, where *Dazl* mRNA expression was consistently detected in gonadal ridges by e12.5 [5, 6]. However, another study using a transgenic mice generated by pronuclear microinjection, in which GFP expression is also driven by the *Dazl* promoter reported that although GFP mRNA was first detected at e12.5, fluorescence could not be observed at this time [16]. *Dazl* expression is known to be maintained through PGCs maturation into gonocytes, and up to spermatogenic cells in adult testis, as also supported by our observations [26]. However, extended *Dazl* expression along the different stages of spermatogenesis is a matter of controversy. *Dazl*-driven expression of GFP in a previous study was only detected from pachytene spermatocyte up to elongating spermatid, while weak expression was detected in some spermatogonia [16]. In that article, non-uniform germ cell staining was described, as some tubules did not stain positive for GFP, and others exhibited partial staining. In contrast, we observed uniform GFP staining in all epithelial cycle stages of the seminiferous tubules, and continued expression from spermatogonia through maturation into spermatocyte, round and elongating spermatid. These discrepancies may be explained by the stronger expression of the reporter gene (GFP) in our model. In agreement, *Dazl* expression was initially described in B-spermatogonia, preleptotene and zygotene spermatocytes, and more intensely in pachytene spermatocyte [2], but later it was shown that *Dazl* also persists in spermatids and even spermatozoa, and that it is expressed in the nucleus of SSCs as well [27, 28]. Recently, it has been reported the existence of an alternatively spliced isoform of murine *Dazl* with a deletion of exon8, referred to as *Dazl_Δ8* isoform, which is expressed in SSCs [7]. We could observe that some cells stained strongly for GFP near the basement membrane of the seminiferous tubules resembling SSCs, but further experiments would be necessary to confirm their identity.

In female gonads, *DAZL* has also been observed in other locations besides germ cell lineage as granulose cells [29], theca interna [30] and *corpus luteum* [31] in human. In mice, *Dazl* expression seems to be limited to the cytoplasm of oocytes and follicular cells in embryonic and prepubertal ovary, and to a peripheral cytoplasmic localization in oocytes from adult ovaries [2]. However, in our murine model, transgene expression was restricted to germ cells as it was only expressed in the cytoplasm of oocytes with no variations from embryonic to adult ovaries.

Although GFP was expressed from sex determination in primordial germ cells through maturation into gametes in testis and ovary, expression levels varied considerably between these tissues and at different stages. This can be explained by the existence of different proportions of germ cells in these tissues, as the percentage of germ cells respect to the total is higher in testicles than in ovaries, and shows variations at different stages. Thus, fetal ovaries contain a higher germ cell: somatic cell ratio than adult ovaries, whereas the opposite situation occurs in testicles. In this regard, in adult ovaries, the low proportion of germ cells did not allow the detection of the transgene by fluorescence stereoscopy. Furthermore, highest *Dazl* expression in females is detected at 17 dpc, when pachytene cells are present in the fetal ovary [2], and transcription levels decrease after birth, as the number of oocytes is depleted [6], which was reflected in our qPCR results.

Dazl expression in other tissues besides gonads is a matter of controversy and raises two questions: 1) Could non-gonadal tissues be a potential germ cell source, and 2) Could *Dazl* have another role distinct to germline development, related to pluripotency. In 2005, Johnson *et al.* reported *Dazl* detection in bone marrow of adult male and female mice, together with other germline markers as *Stella*, *Ifitm3* and *Ddx4*. They also reported oocyte production restoration in sterilized mice by bone marrow and peripheral blood transplantation [11], supporting the hypothesis that non-gonadal tissues could be used as a germ cell source, although this finding has been criticized [32, 33]. Later, another study described that mouse bone marrow mesenchymal stem cells were positive for *Dazl*, but in lower levels of expression than the total bone marrow cells tested [10, 34]. In agreement with these articles, transgene mRNA was detected in total bone marrow cells in our study. Furthermore, transgene mRNA was detected in small and large intestine. Multipotent cells identified in small intestine and in colon crypts [35] could explain *Dazl*-GFP expression. To contrast these observations, *Dazl* mRNA was also detected in bone marrow, as well as in small and large intestine (data not shown). Furthermore, another study reporting *DAZL* location in human amniotic fluid cells [12] indicates that this gene could be expressed in other locations containing adult stem cells. The recently reported alternatively spliced isoform *Dazl_Δ8*is expressed together with the full-length isoform of *Dazl* in different pluripotent cell types: ESCs, mouse adult germline stem cells (maGSCs),

induced pluripotent stem cells (iPSCs), embryonic germ cells (EGCs) and embryonal carcinoma cells (ECCs) [7]. Additionally, another recent study associates *Dazl* with Tet-dependent DNA demethylation in ESCs [36]. Taking into account all these studies, it seems that *Dazl* could have another role distinct to germline development, related to pluripotency.

Supporting this notion, *Dazl* is also expressed in preimplantation embryos. In non-mammal species, *zDazl* has been localized in early zebrafish embryos [37, 38] and *XDazl* in Xenopus embryos until stage 10 [39]. After gastrulation, *XDazl* expression is abolished, and is later turned on in PGCs at the gonadal ridge [40]. In mouse, *Dazl* expression has been reported from zygote to blastocyst [41], playing a role in transcriptional activation necessary for oocyte maturation and early embryonic development [42, 43]. In our transgenic model, *Dazl*-GFP expression could be detected by fluorescence microscopy in preimplantation embryos up to expanded blastocysts. Its expression, not only in inner cell mass cells, but also in trophectoderm cells, might imply other unknown functions of *Dazl*. During postimplantation development, *Dazl* is robustly silenced by methylated CpG-dense promoters to prevent ectopic activation that may drive malignant tumour growth, and it is specifically activated by demethylation in the germline [44]. In agreement, transgene expression was not found by immunohistochemistry in e9.5 (data not shown), but was later observed in e12.5 PGCs by fluorescence microscopy and immunohistochemistry.

A significant difficulty of *in vitro* germ cell generation from ESCs lies in the fact that many PGCs markers are shared with ESCs. Interestingly, several germline-specific genes expressed in ESCs, including *Dazl,* have been found to be comparable to that of pluripotent cells originated from the germ line, i.e. mouse adult germline stem cells (maGSCs). Furthermore, the expression of germ cell markers has been shown to precede pluripotency markers appearance during the time course of iPSCs generation [45]. In fact, we observed robust GFP expression in ESCs and in EBs by fluorescence, in agreement with several studies describing *Dazl* expression in mouse and human ESCs and EBs [8, 9, 25, 46]. Fluorescence was maintained along differentiation through EBs and two different cell populations expressing GFP appeared after EBs differentiation towards germline: PGC-like cells and ESCs. The fact that some ESCs remain in the EBs along differentiation has been previously described [15, 47, 48]. Thus, *Dazl*-GFP expression in EBs is not only restricted to cells committed to a germinal lineage, as fluorescent pluripotent cells remain in the EBs along differentiation.

Conclusions

Our *Dazl*-GFP transgene was expressed *in vivo* in the germline from PGCs through maturation into gametes, and in non-gonadal adult tissues harboring multipotent stem cells, as well as during preimplantation embryo development. *In vitro*, *Dazl*-GFP was expressed in ESCs, in EBs and in PGCs. Thus, our transgene marked both pluripotent and germ cells *in vivo* and *in vitro*, which supports the idea that besides its main function in the germline, *Dazl* plays a role in pluripotency.

List of abbreviations

AP: Alkaline Phosphatase
bFGF: Basic Fibroblast Growth Factor
C_T: Cycle Threshold
Dazl: Deleted in azoospermia like
EBs: Embryoid Bodies
ECCs: Embryonal Carcinoma Cells
eCG: Equine Chorionic Gonadotropin
ESCs: Embryonic Stem Cells
Gapdh: Glyceraldehyde-3-Phosphate Dehydrogenase
GFP: Green Fluorescent Protein
hCG: Human Chorionic Gonadotropin
iPSCs: Induced Pluripotent Stem Cells
LIF: Leukemia Inhibitory Factor
maGSCs: Mouse Adult Germline Stem Cells
MEF: Mouse Embryonic Fibroblast
mRNA: Messenger RNA
PGCs: Primordial Germ Cells
SCF: Stem Cell Factor
SSCs: Spermatogonial Stem Cells

Competing interests

The authors declare that they have no competing interests

Author´s contributions

PRI carried out the genetic expression, immunohistochemistry and immunofluorescence analyses and drafted the manuscript. EP participated in in vitro experiments and reviewed the manuscript. RFG produced the transgenic mice and reviewed the manuscript. AGA designed the study and helped to draft the manuscript. MAR carried out the *in vitro* experiments, participated in the design of the study and reviewed the manuscript. All authors read and approved the final manuscript.

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An efficient system to establish biopsy-derived trophoblastic cell lines from bovine embryos

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Chapter IV / Capítulo IV

Summary

Trophoblastic cells lines provide a useful model to study implantation and placentogenesis. In the same way, these cell lines could reflect the characteristics or alterations carried by the embryos, and could be used as a model for their study. However, only a reduced number of cell lines are currently used for research due to the difficulty to establish new cell lines.

In this chapter we established a system to easily derive new trophoblastic cell lines from bovine embryo biopsies cultured in microdrops in different conditioned media, being the medium conditioned by mouse embryonic fibroblasts the most succesfull. Morphological features and *in vitro* behaviour of the generated trophoblastic cell lines were consistent with those described by other authors. Trophoblast-specific genes were analyzed, and different expression patterns were observed among the cell lines that varied with passages. Moreover, trophoblastic cell lines proliferated for more than two years and expressed different pluripotency-related genes.

Variability in gene expression patterns among trophoblastic cell lines that came from the same derivation conditions could be due to the embryonic source, and variations observed along passages could be caused by long-term culture. Furthermore, bovine trophoblastic cell lines showed certain self-renewal capacity that could reflect the presence of a pluripotent cells population.

Resumen

Las líneas de células trofoblásticas pueden ser usadas como modelos de implantación y placentogénesis. Del mismo modo, estas líneas celulares podrían reflejar las características o alteraciones presentes en los embriones de los que proceden, pudiendo así ser usadas para su análisis. Sin embargo, tan sólo un reducido número de líneas de células trofoblásticas bovinas se utiliza actualmente en investigación debido a la dificultad de producir nuevas líneas.

En este capítulo hemos establecido un sistema eficiente para obtener nuevas líneas celulares trofoblásticas a partir de biopsias embrionarias bovinas cultivadas en microgotas de diferentes medios condicionados, siendo el medio condicionado por fibroblastos embrionarios murinos el más eficiente. Las características morfológicas y el comportamiento *in vitro* de las líneas de células trofoblásticas generadas fueron comparables a lo descrito previamente por otros autores. Además se observaron diferentes patrones de expresión de genes específicos del trofoblasto en las líneas celulares, que también variaron a lo largo de los pases. Las líneas de células trofoblásticas se mantuvieron en cultivo durante más de dos años, y se pudo observar expresión de diferentes genes relacionados con la pluripotencia.

La variabilidad en los patrones de expresión génica observados en líneas de células trofoblasticas obtenidas en idénticas condiciones podría deberse a la fuente embrionaria, y las variaciones observadas a lo largo de los pases podrían estar causadas por el cultivo a largo plazo. Además, las líneas de células trofoblásticas mostraron cierta capacidad de auto-renovación que podría ser reflejo de la presencia de una población de células pluripotentes.

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Abstract

Trophoblastic cells play a crucial role in implantation and placentogenesis and can be used as a model to provide substantial information on the peri-implantation period. Unfortunately, there are few cell lines for this purpose in cattle because of the difficulty of raising successive cell stocks in the long term. Our results show that the combination of a monolayer culture system in microdrops on a surface treated with gelatin, and the employment of conditioned media from mouse embryonic fibroblasts, support the growth of bovine trophoblastic cells lines from an embryo biopsy. Expression profiles of mononucleate (MNC) and binucleate (BNC)-specific genes in established trophoblastic cells lines represented various stages of gestation. Moreover, the ability to expand trophoblastic cell lines for more than two years together with pluripotency-related gene expression patterns revealed certain self-renewal capacity. In summary, we have developed a system to expand *in vitro* trophoblastic cells from an embryo biopsy which solves the limitations of using amplified DNA from a small number of cells for bovine embryo genotyping and epigenotyping, and on the other hand facilitates the establishment of trophoblastic cell lines that can be useful as peri-implantation *in vitro* models.

Introduction

The application of new technologies for genotyping bovine embryos using last generation high-density marker chips or complete genome sequencing, represents an alternative to reduce the generational interval, and to limit the high cost of producing large number of offspring and progeny testing in multi-character genetic selection programs (for a comprehensive review, see [1]). Although these genetic analyses have been successfully performed on embryo biopsies, their main limitation is the reduced number of cells obtained, and therefore the limited amount of genomic DNA as starting material [2]. Preimplantation embryo development is a crucial period in which epigenetic marks are established and persist throughout posterior life. It has been described that epigenetic alterations occasionally occur in embryos produced by assisted reproductive technologies (ARTs) [3] and the identification of early markers of these alterations would avoid posterior epigenetic syndromes or diseases in the animals and would improve livestock efficiency. However, embryo genotyping and epigenotyping present the same critical limitations: reduced sample amount and embryo destruction. To overcome these problems we aimed at developing an efficient system of *in vitro* trophoblastic cell culture from an embryo biopsy which would enable the production of a good quality genomic DNA without pre-amplification.

In cattle, a mononuclear trophoblastic cell (MNC) may differentiate into a binuclear cell (BNC), which then fuses with an endometrial epithelial cell to form a trinucleated cell [4]. In ruminants, the trophectoderm-derived factor, interferon-tau (*IFNT*), produced by MNC, is responsible for sustaining the pregnant state by restricting the pulsatile release of prostaglandin F2α from the endometrial epithelium and thereby stimulating the maintenance of the *corpus luteum* during pregnancy [5][6]. *IFNT* also controls the expression of several uterine-derived factors that prepare the uterus for placental attachment, modifies the uterine immune system, and regulates early *conceptus* development [7]. Therefore, the insufficient production of *IFNT* or the absence of maternal recognition of this signal leads to pregnancy failures in cattle [8]. Moreover, BNCs produce placental lactogen (*CSH2*), prolactin-related protein (*PRPs*), and pregnancy-associated glycoproteins (*PAGs*), among other genes required for placental development [9]. Therefore, trophoblastic cells play a crucial role in implantation and placentogenesis and further analyses would be necessary to understand molecular mechanisms by which trophoblastic-specific genes are regulated.

However, the physiology of trophoblastic cells has remained unclear because of lack of definitive information on cell lineages. Talbot *et al.* [10] derived for the first time two trophoblastic cell lines from the culture of 7-8-days *in vitro*-produced blastocysts (CT-1

and CT-5), using culture dishes containing STO feeder cells. However, the co-culture with mouse feeder cells has the detrimental risk of contaminating the bovine trophoblastic cells, while separation of both cell lines would involve an additional effort. Later, Shimada *et al.* [11] reported the establishment of a bovine trophoblastic cell line derived from *in vitro* matured and fertilized blastocyst (BT-1) in the absence of feeder cells, using bovine endometrial fibroblast-conditioned medium, but attaining very low derivation efficiency. Since then, a large number of studies regarding trophectoderm (TE) signaling and differentiation have been performed with only two trophoblastic cell lines: BT-1 [4][12][13] and CT-1 [14][15][16][17][18]{Michael, 2006, Granulocyte-macrophage colony-stimulating-factor increases interferon-tau protein secretion in bovine trophectoderm cells;Das, 2008, Combinatorial roles of protein kinase A`, Ets2`, and 3'`,5'-cyclic-adenosine monophosphate response element-binding protein-binding protein/p300 in the transcriptional control of interferon-tau expression in a trophoblast cell line;Nakaya, 2009, The characterization of DNA methylation-mediated regulation of bovine placental lactogen and bovine prolactin-related protein-1 genes;Bai, 2011, Regulation of trophoblast-specific factors by GATA2 and GATA3 in bovine trophoblast CT-1 cells;Schiffmacher, 2012, Optimization of a lipitoid-based plasmid DNA transfection protocol for bovine trophectoderm CT-1 cells;Schiffmacher, 2013, CDX2 regulates multiple trophoblast genes in bovine trophectoderm CT-1 cells}. In addition, cotyledonary trophoblastic cell lines have been derived from placental stages [19][20][21]. A larger number of trophoblastic cell lines suitable for *in vitro* studies would be helpful for future studies.

The aim of the present study was to establish an adequate *in vitro* culture system to support the growth of bovine trophoblastic cells from an embryo biopsy using different cell sources of conditioned media, eliminating the risk of contamination with feeder cells. This *in vitro* culture system facilitates the establishment of trophoblastic cell lines, which can be expanded for more than two years and can be useful to study implantation and placentogenesis processes. Moreover, this approach could also be employed to produce a relatively large amount of good quality genomic DNA for bovine embryo genotyping and epigenotyping.

Materials and Methods

In vitro embryo production

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (Madrid, Spain). The techniques for *in vitro* embryo production have been described in detail previously [22]. Briefly, immature cumulus oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of heifers and cows collected at slaughterhouse. COCs were matured for 24 h in TCM-199 supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma, Aldrich, Spain), and 10 ng/ml epidermal growth factor, at 38.5°C under an atmosphere of 5% CO2 in air, with maximum humidity. For *in vitro* fertilization (IVF), matured COCs were inseminated with frozen-thawed Bovi-Pure (Nidacon, Mölndal, Sweden) separated bull sperm at a final concentration of 1 x 10^6 spermatozoa/ml. Gametes were co-incubated at 38.5°C in an atmosphere of 5% CO_2 and maximum humidity. Semen from the same bull was used for all experiments. At approximately 20 h post-insemination (p.i.), presumptive zygotes were denuded and cultured in groups of 25 in 25µl droplets under mineral oil at 38.5°C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The basal medium for all embryo culture was synthetic oviduct fluid (SOF) supplemented with 5% FCS. Cleavage rate was recorded at 48 h p.i. and blastocyst development from day 6 to 10 p.i.

A total number of 9174 *in vitro* matured and fertilized presumptive zygotes were produced in 23 replicates. Zygotes showed a cleavage rate of 85.4±2.1% and a blastocyst yield of 6.5±1.5%, 26.3±2.2%, 28.9±1.7%, and 30.1±1.7% on days 6, 7, 8 and 9 p.i., respectively (mean±SEM). The cumulative hatching rate was 1.1±0.4%, 22.0±3.4%, 46.0±3.3% and 54.0±3.5% on day 7, 8, 9 and 10 p.i., respectively.

Biopsy and embryo survival analyses

Good quality day 8-10 hatched blastocysts were selected under a stereomicroscope based on their morphology according to the criteria of International Embryo Transfer Society (IETS) Manual [23] and placed in groups of five in M2 medium (M7167, Sigma Aldrich Company, Ayrshire, UK) at 38.5°C. Embryo biopsies were performed individually in microdrops of M2. First an incision was made in the plastic surface with a scalpel blade (no. 18) to enable immobilization of the embryo without any bonding substance. Then embryo was cut at the opposed pole to the inner cell mass (ICM) to avoid its damage (Figure 1). Each TE biopsy was washed in corresponding culture medium and transferred for cultivation in microdrop.

For evaluating embryo survival, biopsied embryos (ICM with the rest of TE cells) were washed in SOF medium supplemented with 5% FCS and 5.56 mM glucose, and cultured in

group of 10-15 in droplets of 25 l of SOF under mineral oil for 24 h at 38.5 °C, 5% CO_2, and 5% O_2.

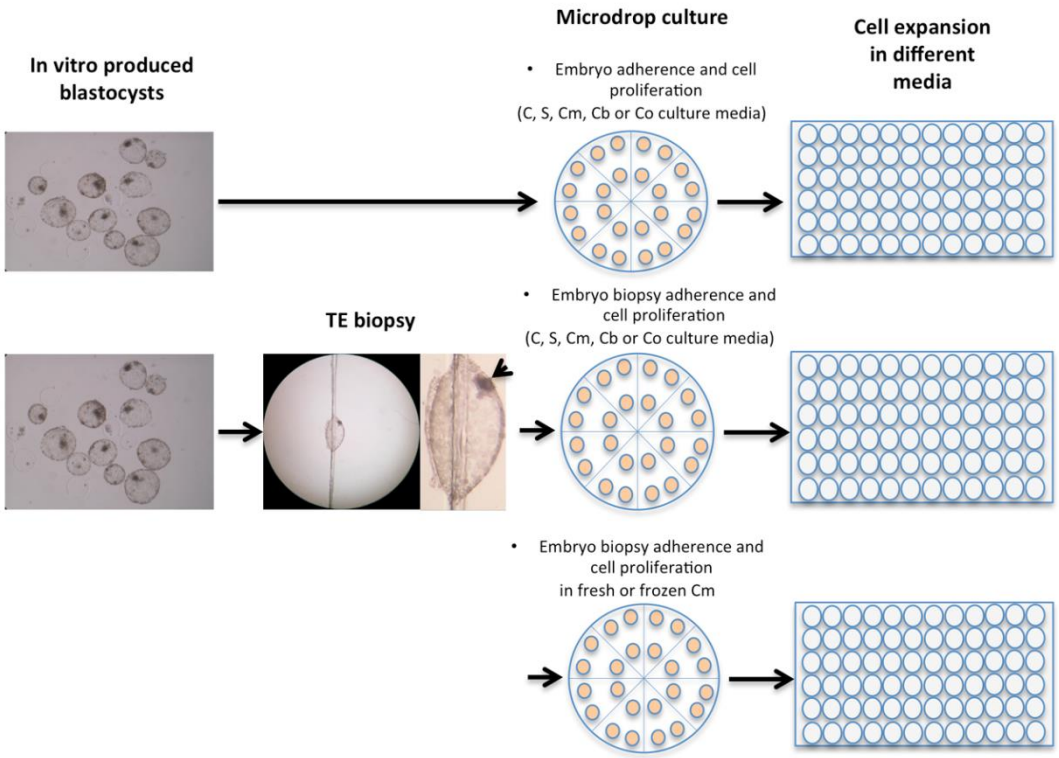

Figure 1. Experimental design. Arrow points to ICM.

Microdrop cultures

Microdrops in corning 35-mm tissue culture dishes (24 x 10-µL droplets) (Figure 1) were coated for 30 min with 5 % gelatin (Sigma Chemical Company, Madrid, Spain). The different media assessed were placed in the droplets under mineral oil and equilibrated for 3 h in the incubator at 38.5°C, 5% CO_2 and saturated humidity. Intact embryos or biopsies were seeded on the microdrops containing the different media.

Preparation of mouse embryonic fibroblast (MEFs)

MEFs were derived from 12.5 day-old foetuses of Swiss mice [24]. The visceral organs and head of the foetuses were removed and the remaining tissue was cut into small pieces. The tissue pieces were incubated in 0.04% (v/v) trypsin-EDTA for 15 min with agitation, and subsequently centrifuged at 110g for 2 min. The supernatants were diluted in control

media consisting of Dulbecco's modified Eagle medium (DMEM) plus 4500 mg/l glucose, glutaMAX, and pyruvate (Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 1 mM MEM nonessential amino acids solution, and an antibiotic mixture containing 100 U/ml penicillin and 100 mg/ml streptomycin, and supplemented with 10% FBS (PAA Laboratories Cölbe Germany) and centrifuged at 300g for 5 min. The pellets were re-suspended in control media for monolayer formation.

Preparation of bovine embryonic fibroblasts (BEFs)

BEFs were derived from foetal skin collected at approximately 2 to 3 months of gestation following the same process described above for MEF isolation.

Preparation of bovine oviductal cells (BOECs)

Ipsilateral oviducts at the mid-luteal phase of the estrous cycle were collected from slaughtered heifers. Each oviduct was flushed with 10 ml of SOF, 5% FCS. BOECs were centrifuged at 750 g for 9 min and the resulting pellet was washed by centrifugation at 100 g for 5 min in 5 ml of the same medium. The final pellet was re-suspended in control medium by pipetting. Oviductal cells were counted in a hemocytometer and diluted in the appropriate volume of culture medium to give a final concentration of $1\mathbf{x}10^6$ cells/ml [25].

Preparation of conditioned media

The media from MEFs, BEFs and BOECs cultures were collected after 72 h of confluent culture, centrifuged at 10000g for 10 min at 4°C, filtered through a 0.22 µm nitrocellulose membrane and stored at -20°C until use. These conditioned media were mixed 1:1 with control media. All media were supplemented with 20 ng/ml of epidermal growth factor (Peprotech, France).

Trophoblastic cell lines long-term culture

Based on our results indicating that Cm is the most appropriate medium for trophoblastic cell lines derivation, long term culture of trophoblastic cell lines was performed in mouse embryonic fibroblasts conditioned medium (Cm) mixed 1:1 with control media, and supplemented with 20 ng/ml epidermal growth factor (Peprotech, France). Passage was done by physical dissociation, removing the monolayer of cells from the tissue culture plate by shooting jets of medium onto the monolayer and shearing by repeated pipette aspirations. Trypsin-EDTA treatment was found to be deleterious to the cells. Trophoblastic cells lines were routinely plated at a 1:2 dilution from confluent cell culture every 7-10 d.

Cell count of biopsies and biopsied embryos

Biopsies and biopsied blastocysts were allowed to recover for 3 h after the biopsy in SOF + 5% FCS and 5.56 mM glucose, and stained with Hoechst 33342 (10 mg/mL in 2.3% (w/v) sodium citrate) to be visualized in an epifluorescence microscope.

Mononucleated and binucleated cell counting

For cell count in a monolayer, glass coverslips were coated with 5% gelatin (Sigma, Madrid, Spain) and placed into a tissue culture plate to allow trophoblastic cells attachment and growth. Once the coverslips were covered by a confluent monolayer, cells were washed with PBS and fixed with 4% paraformaldehyde (Panreac, Barcelona, Spain) for 1 h. Samples were incubated with 5 µg/ml Hoechst 33342 (Sigma, Madrid, Spain) in PBS for 15 min in the dark and washed with PBS. Then, glass coverslips were removed from the tissue culture plates and mounted with a 1:1 PBS: Glycerol (Sigma, Madrid, Spain) solution containing 1 µg/ml Hoechst 33342 onto slides to be visualized in an epifluorescence microscope.

For cell counts in spheroids, they were collected from the supernatant of TE culture dishes, washed with PBS and fixed with 4% paraformaldehyde (Panreac, Barcelona, Spain) for 1 h, washed with Tris-buffered Saline and permeabilized by incubation with 0.5% Triton X-100 for 5 min. Samples were stained overnight with AlexaFluor488-Phalloidin (Invitrogen) and mounted with Prolong (Invitrogen) supplemented with DAPI (1µg/ml). Images were acquired in a Leica TCS-SP5 confocal laser-scanning unit equipped with Ar and He/Ne laser beams and attached to a Leica DMIRBE inverted epifluorescence microscope (Leica Microsystems, Heidelberg, Germany) [26].

TUNEL apoptosis assay

The TUNEL assay for apoptotic cell detection was performed using the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions with minor modification [24]. Briefly, three confluent cell plates of each trophoblastic cell line were fixed with 4% Paraformaldehyde in PBS for 1 h at room temperature. Then, cells were incubated in permeabilization solution containing 0.1% Triton X-100 in PBS for 15 min at 37 ºC, followed by incubation in TUNEL reaction mixture for 1 h at 37ºC. Finally, a solution containing 1 µg/ml Hoechst 33342 was added and cells were analyzed by fluorescence microscopy. The number of TUNEL positive cells in four different microscopic fields was counted, and apoptotic index determined by calculating the ratio of the TUNEL positive cells/total cells.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Poly (A) RNA was extracted from ten different trophoblastic cell lines using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech) following the manufacturer's instructions with minor modifications [27]. Immediately after extraction, the reverse transcription (RT) reaction was carried out following the manufacturer's instructions (Epicentre, Madison, WI, USA). Briefly, OligodT primer and random primers (Biotools, Madrid, Spain) were added to the samples and heated to 70°C for 5 min to denature the secondary RNA structure. Then, the RT mix was completed by the addition of 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNAsin RNAse inhibitor (Promega, Madison, WI, USA), MMLV HP RT 10X Reaction Buffer, 5 mM DTT and 50 U MMLV High Performance Reverse Transcriptase (Epicentre, Madison, WI, USA). Tubes were incubated at 42°C for 60 min to allow the reverse transcription of RNA, followed by incubation at 70°C for 10 min to denature the RT enzyme [28].

After reverse transcription, PCR amplification was performed by adding a 2-µL aliquot of each sample in a total volume of 25 µL of PCR mix containing 10 µM specific primers, 0.1 mM dNTPs (Biotools, Madrid, Spain), 2 mM $MgCl_2$, 10X Buffer and 1U Biotools DNA Polymerase (Biotools, Madrid, Spain). For *PAG1*, *CSH2* and *PRP1* amplification, heminested and nested PCRs were performed by adding a 2-µL aliquot of PCR product in a total volume of 25 µL of PCR mix containing 10 µM specific primers, 0.1 mM dNTPs (Biotools, Madrid, Spain), 2 mM $MgCl_2$, 5X GoTaq Flexi Buffer and 1U GoTaq DNA Polymerase (Promega, Madison, WI, USA). Primer sequences and amplification temperatures are listed on Supplementary Table 1. The PCR products were subjected to electrophoresis in a 2% agarose gel and recognized by ethidium bromide staining.

Supplementary Table 1. Details of primers used for gene expression analysis

Gene	Forward / Reverse	Annealling Temperature	NCBI Reference	Product size
H2AFZ	F-AGGACGACTAGCCATGGACGTGTG R-CCACCACCAGCAATTGTAGCCTTG	54 ºC	NM_174809.2	209 bp
AMELX / AMELY	F-CCAGCCAAACCTCCCTCTGCC R-CCCGCTTGGTCTTGTCTGTTGC	60 ºC	NM_001014984.1 /NM_174240.2	217 / 280 bp
CDX2	F-ATCACCATCCGGAGGAAAGC R-CTCATGGCTCAGCCTGGAAT	54 ºC	NM_001206299.1	333 bp
ELF5	F-GAAGGCTGAACAGAGGTGCC R-TGCTGTGTGTCACTGAGTCC	59 ºC	NM_001024569.1	149 bp
IFNT	F-TCCCCATGGCCTTCGTGCTCTCTCT R-CTCAAAGTGAGTTCAGATCTCCACC	54 ºC	NM_001015511.3	593 bp
POU5F1	F-CGAGTATCGAGAACCGAGTG R-CAGGGTTCTCTCCCTAGCTC	54 ºC	NM_174580.2	440 bp
CDH1	F-GACACTGGAGGTATCAGCGCAC R-TGATCTGGACCAGCGACTTAGG	54 ºC	NM_001002763.1	193 bp
TERC	F-GGTGTCCATTGCCGCCGTGA R-TCTGAGCCGAGTCCCGGGTG	54 ºC	NR_001576.1	160 bp
TERT	F-TGTCTGACGCCCAGGCGCT R-GATGAAGGTGCAGGACTGCG	54 ºC	NM_001046242.1	240 bp
FGF4	F-AACGTGAGCATCGGCTTCCACC R-TTGCTCAGGGCGATGAACATGC	54 ºC	NM_001040605.2	284 bp
***CSH2* 1st**	F1-TGCCACACCGAATTCATGAC R2-AGGGCTTCGTCCTCTGTATTTG	50 ºC	NM_181007.2	186 bp
***CSH2* 2nd**	F3- TGCACAGGAATGGAGCCTCACC R4-TGGCGCACATCCTCATCGTCTG	54 ºC		
***PRP1* 1st**	F1-CACGGAGCTGCAGCATATGA R2-CCTTGTGGCGCTTGATAGGA	48 ºC	NM_174159.2	196 bp
***PRP1* 2nd**	F3-TCCTGCGGTCCTGACGTGTTTG R4-TCGGGAGCATGGAAGGAATTGG	56 ºC		
***PAG1* 1st**	F1- CAACGTGCCATTTCTGAGCCTG	54 ºC	NM_174411.2	145 bp
***PAG1* 2nd**	F3-ATCGGTGCCATACCACGGG R-AGCAGCGGCCTCTATCATC	50 ºC		

Trophoblastic cell lines sex determination

DNA was obtained from trophoblast cell lines by phenol/clorophorm extraction and sex was determined through the bovine X/Y amelogenin polymorphism by PCR. Primer sequences and amplification temperature are listed in Supplementary Table 1. The PCR products were subjected to electrophoresis in a 2% agarose gel and recognized by ethidium bromide staining. The gel was visualized under ultraviolet light to identify the specific bands. A 280 bp band was observed in female cell lines and 280 bp plus 217 bp bands in male cell lines [29].

Results

Embryo adherence and cell proliferation

A rapid adhesion of bovine embryos is a critical factor for cell proliferation and survival *in vitro.* Initial adhesion was improved when cells were placed in a small 10µl microdrop under mineral oil on previously coated with gelatin tissue culture dishes, without the use of a feeder layer. Under these conditions, over 70% of intact bovine embryos were able to attach and cell proliferation could be clearly observed as early as 4 days of culture (Table 1).

To determine the optimal medium for adherence and growth of intact bovine embryos in the absence of feeders, we compared the following media: (i) Control medium supplemented with 10% FCS (C); (ii) SOF supplemented with 5% of FCS (S); and three conditioned media at a rate of 1:1 with control medium: (iii) from mouse embryonic fibroblasts (Cm); (iv) from bovine embryonic fibroblasts (Cb) and; (v) from bovine oviductal cells (Co) (Figure 1). All media used were also supplemented with 20 ng/ml of epidermal growth factor (Peprotech, France).

Adherence accompanied by cell proliferation was assessed at day four in a total of 216 day 8-10 hatched blastocysts. The highest rate of proliferation was obtained in the Cm (100±0.0%) and Co (97.8±2.2%) groups, which were significantly different to C (87.0±4.2%), Cb (66.7±3.0%) and S (79.1±3.6%) (Table 1).

Those lines that reached cell confluence on days 7-10 were expanded to 96-well culture dishes. Cultures were released from the microdrops and transferred to new 96-well culture dishes. The culture media with significantly higher efficiency of proliferation from expansions were Cm (100±0.0%), and Co (81.8±1.6%)(Table 1).

Table 1. Embryo adherence and cell proliferation in different media.

	Culture media				
	C	S	Cm	Co	Cb
N	48	71	25	39	33
Proliferation µdroplet (4d)					
n	42	56	25	38	22
%(Mean±SEM)	87.0±4.2^{c}	79.1±3.6^{bc}	100.0±0.0^{a}	97.8±2.2^{a}	66.7±3.0^{b}
Proliferation P96 (reexpansion, 10d)					
n	21	0	25	31	7
%(Mean±SEM)	48.9±5.1^{b}	0.0±0.0^{b}	100.0±0.0^{a}	81.8±1.6^{a}	31.6±3.0^{b}

^{abc} P<0.05. Values with different superscripts letter in the same row are significant different. One-way ANOVA. Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package.

Embryo biopsy and survival

Our next goal was to isolate trophoblastic cells by biopsy from bovine embryos without affecting the final survival of the remaining embryo. Each TE biopsy was transferred for cultivation in microdrop on gelatin-coated surfaces, as described above for full embryos (Figure 1). To determine the rate of in vitro embryo survival post biopsy, TE biopsies were performed in six replicates from a total of 190 grade 1 and 2 Day-8 hatched blastocysts, based on the guidelines of the International Embryo Transfer Society [23], showing a survival rate at 24 h post biopsy of 82.2±2.6% (mean±SEM). 16 h after biopsy, the biopsied blastocysts (which included ICM) re-expanded and recovered their typical morphology (Figure 2a). TE biopsies (devoided of ICM) also re-expanded and adopted a round morphology 16 h after the biopsy procedure (Figure 2b).

From a total of 25 grade 1 and 2 Day-8 hatched blastocysts, produced in six replicates, cell count on biopsies and biopsied embryos showed an average of 72.44±3.82 cells (42.37% of total cells), and 98.52±3,27 cells (57.63% of total cells) respectively, while the average number of total cells in an intact embryo was 170.96±4,59 (mean±SEM).

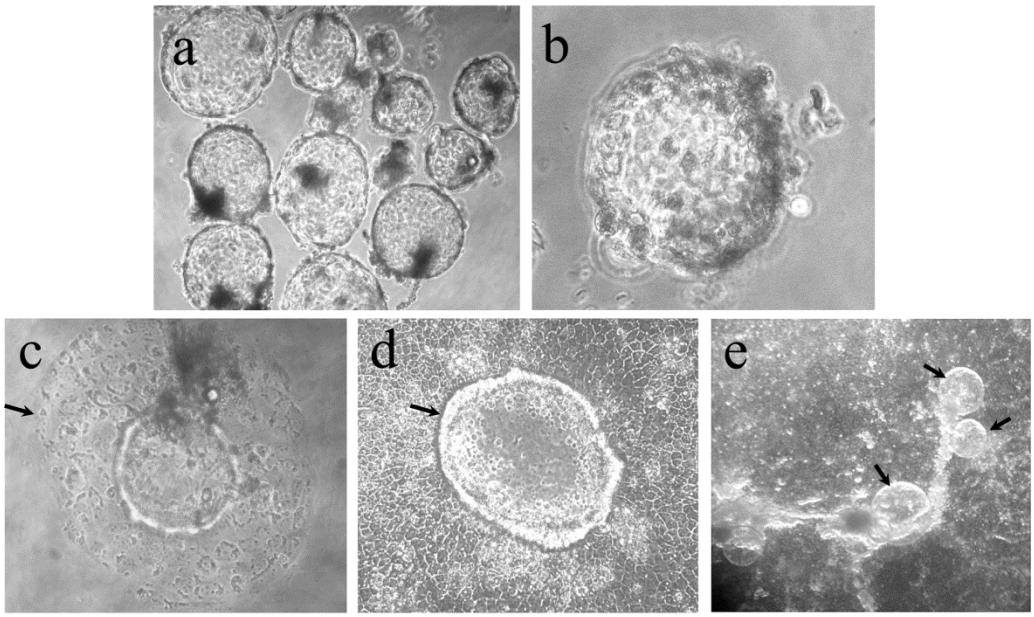

Figure 2. Phase-contrast micrograph examples of (a) biopsied embryos after 16 h of culture, and (b) TE biopsies after 16 h of culture. Magnification ≈ 30x. (c) Phase-contrast micrograph examples of IVF bovine blastocyst's primary trophoblastic outgrowth only 48 h after initiating the culture without feeder layer. Magnification 200x. Arrows indicate the borders of the primary colony. (d) Trophoblastic cell cultures after 7-10 days. Note dome formation (arrow) in the monolayer. Magnification 200x. (e) Trophoblastic cell culture after 21 months and 76 passages. Magnification 100x. Note cavern formation (arrow).

Trophoblastic cells adherence and proliferation

We next evaluated the efficiency attained in our different media conditions of the adhesion and proliferation of trophoblastic cells derived from biopsies cultured on gelatin-coated microdrops. A total of 221 TE biopsies from hatched day 8-10 blastocysts were used. The greatest proliferation was again registered when Cm was used (73.9%) (Table 2). We could detect adhesion and proliferation of some biopsies as early as 48 h of culture (Figure 2c). Regarding the expansion of biopsy cultures in P96, we observed no differences between C (55.6±5.6%), Cm (48.2±4.3%) and Co (43.8±3.4%) groups; while Cb group presented a significantly lower percentage of expansion (30.0%) and S did not support any expansion of the biopsy (0.0%) (Table 2).

Table 2. TE biopsy adherence and cell proliferation in different media.

	Culture media				
	C	S	Cm	Co	Cb
N	35	42	42	69	33
Proliferation µdroplet (4d)					
n	18	15	31	41	13
%(Mean±SEM)	51.4±1.52[b]	35.7±2.7[c]	73.9±1.6[a]	60.3±1.5[b]	39.4±3.0[c]
Proliferation P96 (reexpansion 10d)					
n	10	0	15	18	4
%(Mean±SEM)	55.6±5.6[a]	0.0±0.0[b]	48.2±4.3[ac]	43.8±3.4[ac]	30.0±21.5[c]

[abc] P<0.05. Values with different superscripts letter in the same row are significant different. One-way ANOVA. Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package.

Because we may loose the activity of relevant soluble factors by freezing/thawing the conditioned medium, we analyzed proliferation in the presence of fresh Cm conditioned media, finding no differences between fresh or frozen Cm media at day 4, when microdrop confluence was reached (71.0±4.9% vs 77.9±4.1% respectively), or at day 10 of culture in P96 (44.8±8.8% vs 57.6±8.3).

Ten trophoblastic cells lines were routinely plated at a 1:2 dilution from confluent cell culture every 7-10 d and three of these lines have been cultured for more than 80 passages over two years maintaining trophoblastic morphology and steady proliferation rate (Figure 2e). Thus, trophoblastic cells are apparently capable of an unlimited number of population doublings.

We observed that trophoblastic cell lines in continuous culture showed an accumulation of fluid under the monolayer that elicited the formation of dome-like structure within the colony (Figure 2d) [11]. Such domes continued to accumulate fluid until discrete vesicles were formed (Figure 2e). Subsequently, vesicles spontaneously dissociated from the colony and formed spheroids, which were released to the medium. These spheroids of trophoblastic were able to attach within 24 h of being transferred to a new dish, giving rise to new trophoblastic cells outgrowths.

To analyze spheroids, cell-cell boundaries were visualized by phalloidin staining, which labels the cortical actin cytoskeleton (Figure 3a). In the three-dimensional optical sectioning of the spheroids, we could clearly observe that cells formed discrete layers of

polarized cells. Some spheroids were quite complex in their topology, and frequently presented several cell layers (Figure 3a).

We next assessed whether TE lines recapitulated the ability of producing binucleated cells, by counting mononucleated and binucleated cells in both monolayer cultures and three-dimensional spheroids. In both cultures, the majority of cells of the trophoblastic cell lines were found to be mononucleated, but 2-9% of binucleated cells were found in spheroids and 8-12% on monolayer cell cultures (Figure 3b).

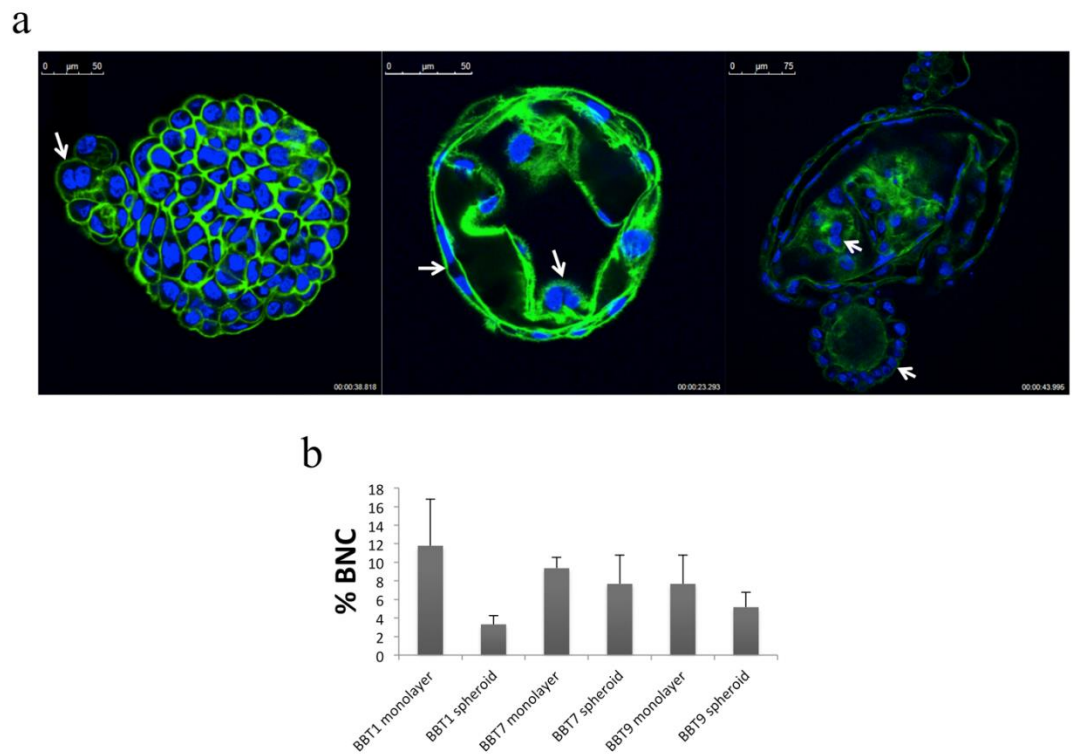

Figure 3. (a) Representative confocal image of the phalloidin/DAPI staining used for BNC cell counting in spheroids. Arrow points to a binucleated cell. (b) Proportion of binucleated cells corresponding to spheroids from different trophoblastic cell lines (Mean±SD of over 6000 cells counted in three replicates).

Apoptotic cells were rarely detected in these culture conditions, ranging from 0.45±0.37 to 3.04±1.86 % in the different TE lines (Figure 4). Only when cell cultures were too confluent abundant apoptotic cells were detected in the wrinkled edges of the monolayer.

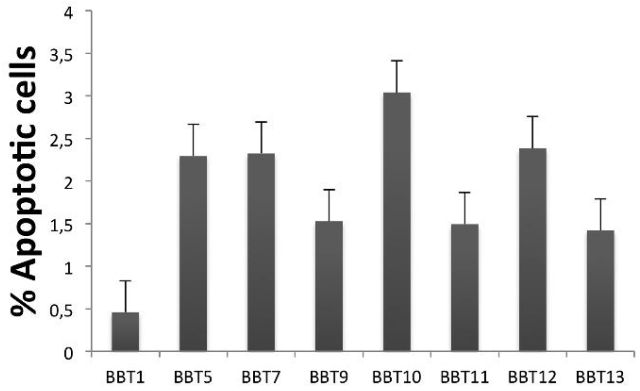

Figure 4. Apoptotic cell detection by TUNEL assay in different trophoblastic cell lines. At least 5000 cells were counted for each cell line.

Sex of trophoblast cell lines was determined through the bovine X/Y amelogenin polymorphism by PCR [29]. A 280 bp band was observed in female cell lines (BBT7, BBT9, BBT10 and BBT12), and 280 bp plus 217 bp bands in male cell lines (BBT1 and BBT11).

Trophoblastic cells gene expression

The expression of early trophoblastic markers (*CDX2* and *ELF5*), and pregnancy-related genes (*IFNT* produced by MNC, and *PAG1*, *PRP1* and *CSH1* produced by BNC) was analyzed in six trophoblastic cell lines at different passages. Lines BBT10, 11 and 12 were analyzed at early passage (p3), and at medium passage (p20); and lines BBT1, 7 and 9 were analyzed at medium passage (p30) and at late passage (p75) (Figure 5). A wide range of gene expression patterns was observed among the cell lines, and gene expression also varied along time in culture.

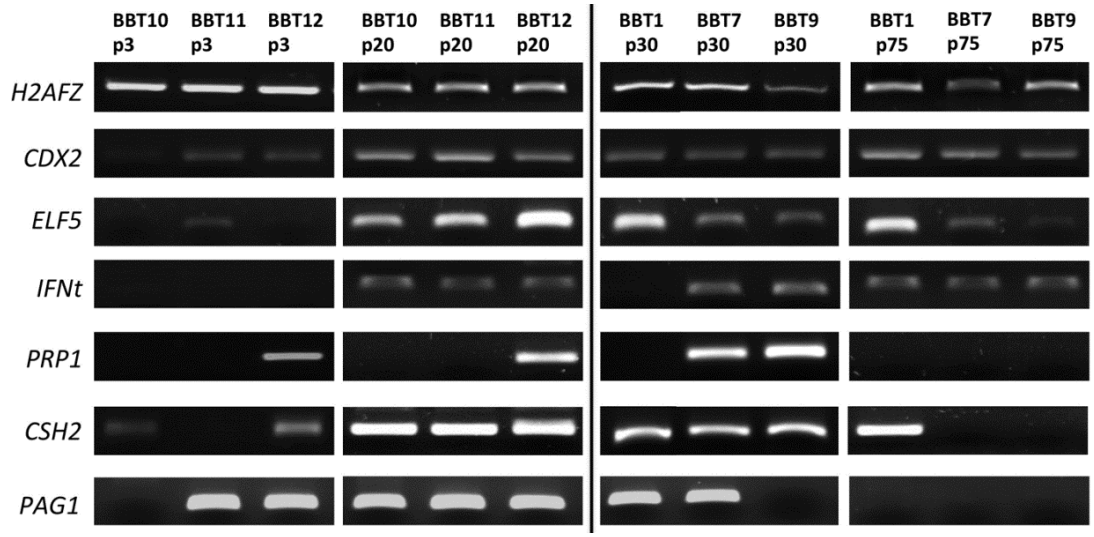

Figure 5. Trophoblastic-specific genes expression in diverse trophoblastic cell lines at different passages.

Pluripotency-related genes: POU class 5 Homeobox 1 (*POU5F1*), Cadherin 1 (*CDH1*), Telomerase RNA Component (*TERC*), Telomerase Reverse Transcriptase (*TERT*) and Fibroblast Growth Factor 4 (*FGF4*) expression was also analyzed at the same passages in six trophoblastic cell lines (Figure 6). In the same way, different expression patterns were found that varied with time in culture.

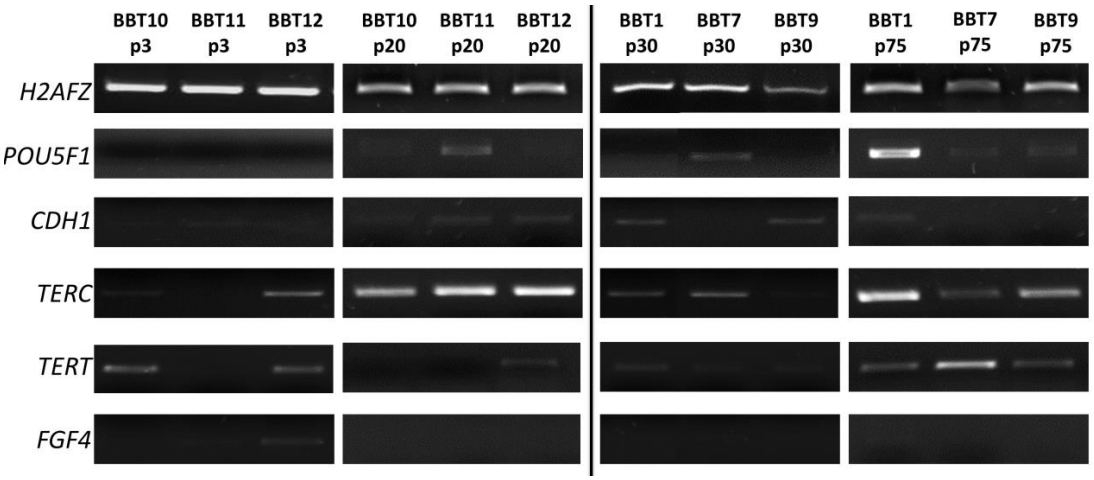

Figure 6. Pluripotency-related genes expression in diverse trophoblastic cell lines at different passages.

Discussion

In this manuscript we describe an efficient method to obtain a trophoblastic cell line from a single bovine embryo or from an embryo biopsy.

Using amplified DNA from embryo biopsies for genotyping has many technical limitations as reduced genome coverage, allele drop-out at heterozygous loci, missing genotypes, amplification of artifacts or allele drop-in [2]. Likewise, little is known about preimplantation embryo epigenetics due to the technical limitations derived from analyzing limited sample amounts. Our procedure for *in vitro* trophoblastic cell expansion represents a suitable new method to produce sufficient amount of genomic DNA for the analysis of multiple production traits with economic interest, overcoming the drawbacks of DNA pre-amplification. In the same way, our system would allow the analysis of the epigenetic profile of preimplantation embryos in order to correlate genome-wide epigenetic characteristics of TE biopsies with epigenetic alterations that could have a short-term effect in implantation and fetal development, or a long-term effect during adulthood. Although additional time is required for the culture step in our system, it is an easy and reliable system and we estimate that in 10 days we can increase the cell number up to 30000 and it is a reliable approach to obtain abundant genomic DNA.

In addition, few trophoblastic cell lines have been derived in cattle due to the difficulties found during the long-term culture, so the establishment of different trophoblastic cells lines provides a valuable tool to explore peri-implantation and placentation processes in ruminants.

Regarding our methodology, our IVF results showed an average blastocyst yield of around 29.0% between days 7 and 10, being within the average range of *in vitro* bovine embryo production [25]. Our hatching rate of 54.3% on day 10 with the largest proportion of hatched blastocysts occurring on days 8 and 9 is also consistent with previously reported data for good quality embryos [30] [31]. Embryo biopsy is a reliable technique as previous reports attained survival rates of 90% 24h after embryo biopsy using micro-section of an embryo on day 7 [32] [33]. Survival rate and embryonic development after the biopsy were identical between embryos produced *in vitro* or *in vivo* [33]. We achieved a survival rate of 82.2% of biopsied embryos, confirming that the chosen state (hatched blastocysts on day 8-10) and quality of the embryos was adequate. Even though the site of the biopsy has been shown to directly affect the quality of the embryos in terms of survival [32], our method did not damage the embryo even when 40% of the total cells were biopsied, since the biopsy was always performed at the opposing pole of the ICM.

Regarding bovine trophoblastic cell lines, a very limited number have been reported so far. Shimada et al [11] started from more than 50 blastocysts, and within a week only a small proportion attached and began to spread out, while the majority neither attached nor grew. They finally obtained only one trophoblastic cell line in absence of feeder cells, using bovine endometrial fibroblast-conditioned medium. The relevance of our work lies in the possibility of establishing a trophoblastic cell line from each embryo, with 100% efficiency in Cm medium. Previous attempts to culture biopsied cells from bovine blastocysts were reported by Le Bourhis et al [34] and by Gamarra et al [35]. However, none of the biopsied cells attached to the coated dishes, probably because they had a very low number of cells (from 5 to 10), large culture surfaces were used (4-well culture dishes) or the media used was not adequate. We found that a rapid adhesion of biopsied trophoblastic cells is a limiting factor. This step was greatly enhanced when using 10µl droplets on previously gelatin-coated surfaces under mineral oil. In the case of biopsies, adhesion began only 48 h after initiating the culture (Figure 2c), and conditioned media from mouse embryonic fibroblasts supported the growth of 78% of TE biopsies. Once cell confluence was reached in microdrops (≈3000 cells) at 7-10 days we were able to re-expand these cultures to P96 plates. From all the initiated trophoblastic cell cultures, 57% reached cell confluence (≈30000 cells) in P96 re-expansions. The reduction in the efficiency in trophoblastic cell lines establishment starting from biopsies (57%,) compared with the 100% proliferation in the case of complete embryo culture, could be explained by the reduction in the number of starting cells [36].

We supplemented all media with EGF, since earlier studies demonstrated its positive effect on proliferation of trophoblastic cells [19]. In a recent study, Suzuki et al. used BMP4 to raise trophoblastic cell lines because exogenous BMP4 stimulated embryonic stem cells (ES) to become trophoblastic cells [37] but no significant differences were found between untreated and BMP4-treated groups regarding derivation efficiency.

Most of the studies that aim to investigate the *in vitro* process of placentation have been performed with cell lines established by co-cultivation with mouse feeder layers [10][38][39]. In our study, cell lines were established from mouse fibroblast conditioned media, in the absence of feeder layer, eliminating the risk of contamination of the extracted genomic DNA. Endoderm outgrowths have also been shown to frequently contaminate the primary trophoblastic cell cultures [10, 38], as well as some condensed and dark cell clumps resembling the ICM [40]. Our methodology avoids contamination by other cell types because only trophoblastic cells are included in the TE biopsy.

The media conditioned from bovine oviductal cells (Co), bovine embryonic fibroblasts (Cb) as well as control media (C) allowed the establishment of cell lines, although the initial growth from the biopsy was significantly lower than in the case of media conditioned by

mouse embryonic fibroblasts (Cm). Therefore Cm is an optimal medium for rapid adhesion and bovine trophoblastic cell growth.

The morphological features and *in vitro* behavior of the different bovine trophoblastic cell lines that we have established are consistent with those described by Shimada et al [11]. We observed that trophoblastic cell lines in continuous culture formed dome-like structures that maturated into vesicles, which spontaneously dissociated from the colony forming spheroids that floated freely in the medium. *In vivo*, trophoblastic binucleated cells are present at the beginning of implantation and throughout pregnancy, representing about 20% of trophoblastic cells [41]. However, it has been described that the availability of BNCs *in vitro* is limited (2-3%) [4] and that their secretory activity cease within several days in culture [42]. In contrast, in our culture conditions a higher proportion of BNC appeared. The expression of *PRP1*, *CSH2* and *PAG1* confirms the existence of binucleated cells in our trophoblastic cell lines, and the expression of these BNC-specific genes was maintained after more than 80 passages.

Thus, our culture system of individual biopsy-derived trophoblast cell lines can serve as a model for the investigation of placental development and the differentiation of the bovine embryo *in vitro*.

The different trophoblastic cell lines established showed diverse gene expression profiles of early trophoblastic markers (*CDX2, ELF5*), MNCs (*IFNT*) and BNCs (*PAG1, PRP1* and *CSH2*) specific genes that varied along time in culture. Differences in gene expression patterns could be due to the variability among the starting embryos, as gene expression in early passages varied among trophoblastic cell lines coming from the same culture conditions; but also to time term in culture, as variations were observed along passages, indicating that these cells lines are dynamic populations.

Among the genes studied, *CDX2* first specifies the trophoblast *versus* the ICM cell fate [43] and is a core regulator of multiple trophoblast genes in bovine trophoblastic cell line CT-1 [18]. In fact, we could observe *CDX2* expression in all trophoblastic cell lines at different time points. *ELF5* transcription factor is detected in bovine blastocysts at the ovoid stage, peaking at day 12 [43] and decreasing during the elongation process [44]. In our study, *ELF5* was expressed in every trophoblastic cell line, mostly from passage 20 onwards. Its expression was also described in CT-1 cell line, which was compared to the ovoid trophoblast stage [18].

Expression of interferon-t (*IFNT*), placental lactogen (*CSH2*), prolactin-related protein 1 (*PRP1*) and pregnancy-associated glycoprotein 1 (*PAG1*) is necessary for pregnancy establishment in cattle. The transcription of these genes is regulated in a temporal and

spatial manner by not completely elucidated molecular mechanisms. *IFNT* is expressed from the formation of the TE in the early blastocyst until its attachment to the maternal uterus after day 19 of pregnancy in cattle [45]. Different factors have been shown to influence *IFNT* expression. Long time in *in vitro* culture of blastocyst or trophoblastic cell lines has been negatively correlated with *IFNT* secretion [46], although in our trophoblastic cell lines *IFNT* expression was detected up to passage 75. Sexual dimorphism affects *IFNT* expression as well, since female blastocysts have been shown to produce twice as much *IFNT* as male blastocysts [47], but we could not find any correlation among sex and *IFNT* expression in our trophoblastic cell lines.

The co-expression of early and late trophoblastic markers would make difficult to associate our trophoblastic cell lines to a specific gestational stage. However, the fact that trophoblastic cell lines express simultaneously early and late trophoblast specific-markers has been previously described [40].

It has been suggested that bovine trophoblastic cells are not terminal cells and present some stem cell features [40]. Therefore, we assessed pluripotency-related gene expression. Telomerase is the enzymatic complex responsible of adding telomeric repeats to the ends of chromosomes. Its expression is silenced in adult somatic tissues with the exception of adult stem cell compartments [48]. The core telomerase components are the telomerase reverse transcriptase (*TERT*), and the telomerase RNA (*TERC*) [49]. Acquisition of indefinite self-renewal capacity in reprogrammed cells is accompanied by induction of *TERT* and *TERC* genes, which is considered a hallmark of the pluripotent state [50] [51]. In our study, *TERC* and *TERT* expression was observed in almost all trophoblastic cell lines generated, which could be an indicator of self-renewal capacity.

POU5F1 expression is rapidly down-regulated in the TE during blastocyst stage in mouse [52]. In contrast, in bovine, *POU5F1* expression is maintained through the ovoid-stage TE [53], and may play a role in maintaining TE cells in a “differentiation-delayed” state [43]. *POU5F1* expression has been reported in CT-1 trophoblastic cell line [18] and in other trophoblastic cell lines generated afterwards, although cells expressing *POU5F1* were rare (only about 4% [40]). In our study, *POU5F1* expression was detected in some trophoblastic cell lines, mainly at medium and late passages, which could be an indicator of cell line immortalization.

In recent years, E-cadherin (*CDH1*) has been shown to regulate pluripotent and self-renewal signaling pathways in stem cells. Loss of E-cadherin is associated with ES differentiation and its expression enhances iPS cell derivation [54]. *CDH1* was expressed in BBT1, BBT5 and BBT9 cell lines. In addition, *FGF4*, produced by the ICM and to a lesser extent by TE in ruminant [55], an inductive signal for trophectoderm elongation in porcine

embryos [56], and an essential factor for mouse trophectoderm stem cells proliferation in an undifferentiated state [57] was expressed in BBT11 and BBT12 cell lines, although expression was lost with time in culture. Thus, the expression of different pluripotency-related genes, together with long-term steady cell proliferation, indicates certain self-renewal capacity of the trophoblastic cell lines generated in this study.

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General discussion

Over the last few decades, much research has been done on pluripotent cells; however, optimal conditions for identification, isolation and *in vitro* culture of pluripotent cells remain unknown. Thus, several determinant aspects for cell lines derivation, as the effect of embryonic source or culture conditions, have been analyzed in this thesis. Furthermore, an efficient system for trophoblastic cell lines derivation from embryo biopsies has been established that allows the development of placentation studies and preimplantational genetic and epigenetic diagnosis.

Embryonic source affects pluripotent cell lines derivation

ESCs have been the most commonly used pluripotent cell type since they were first isolated (Evans and Kaufman 1981). ESCs isolation techniques and culture systems have evolved in the last years to improve derivation efficiency, but it remains controversial whether embryonic characteristics have an influence over the process.

ARTs as *in vitro* culture (IVC), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) or somatic cell nuclear transfer (SCNT) have contributed to the development of infertility treatments in humans and of new animal reproduction methods. However, embryo manipulation and *in vitro* culture is associated with a number of perturbations of the embryonic ultrastructure, genetic and epigenetic alterations and syndromes and diseases during adulthood (Rizos, Fair et al. 2002, Corcoran, Fair et al. 2006, Fernandez-Gonzalez, Moreira et al. 2008, Calle, Miranda et al. 2012). Following ARTs, the classification of good quality embryos is based largely on subjective criteria. Furthermore, it is often overlooked that human ESCs are generated from *in vitro* cultured, often surplus or discarded embryos considered unsuitable for transfer in infertility clinics. These circumstances lead to the question of whether ESCs retain some memory of the embryos from which they were derived.

In chapter I, we have performed ICSI with DNA fragmented sperm (DFS), a previously delevoped model in our laboratory (Fernandez-Gonzalez, Moreira et al. 2008), which can be used as a model to produce bad quality embryos, and we have demonstrated that these embryos show a reduced potential to generate ESCs lines compared to *in vivo*-produced embryos. Furthermore, during early passages, these DFS-ICSI ESCs differ from *in vivo*-ESCs in their expression of certain genes related to pluripotency and epigenetic repression, DNA damage and repair, and *de novo* DNA methyltransferases and histone deacetylases. However, at late passages no differences in gene expression were observed. This is consistent with a previous study that described the retention of epigenetic differences in ESCs dependent on the *in vivo* or *in vitro* fertilization (IVF) origin of the embryo from which they were derived, and the disappearance of these differences after several passages (Horii, Yanagisawa et al. 2010). Another study describing

differences at the transcriptional level between ESCs derived from *in vivo* and *in vitro* cultured (IVC) embryos suggested that these differences are stably maintained during long-term culture (Harvey, Mao et al. 2012). However, the theory that pluripotent cells of different origins would eventually adopt similar genetic and epigenetic profiles after several passages has been previously demonstrated in ESCs (Ramirez, Pericuesta et al. 2007, Horii, Yanagisawa et al. 2010) and in iPSCs (Polo, Liu et al. 2010).

Gene expression differences observed in DFS-ICSI ESCs at early passages could reflect the alterations previously described in the embryo and in adult animals (Fernandez-Gonzalez, Moreira et al. 2008). Epigenetic alterations previously reported in DFS-ICSI embryos could be related to the down-regulation of both histone deacetylase *Hdac10* and *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* observed in the DFS-ICSI ESCs lines. Furthermore, it has been recently described an alternatively spliced variant of *Dnmt3b* lacking exon 6 (*Dnmt3bΔ6*) that has a lower activity than normal *Dnmt3b,* and induces hypomethylation status. *Dnmt3bΔ6* is specific to mouse IVC embryos and is transmitted to ESCs derived from them (Horii, Suetake et al. 2011). Premature aging observed in DFS-ICSI animals could be related to the down-regulation of the repair enzyme-coding *Alkbh3* (Morita, Nakane et al. 2010) and to the higher expression of *Gadd45* (Moskalev, Smit-McBride et al. 2012) observed in the DFS-ICSI ESCs lines at early passages.

Furthermore, pluripotency in the DFS-ICSI ESCs lines was affected, as lower *Sox2* expression and reduced derivation efficiency were observed. In contrast, Horii *et al*. reported no differences in derivation efficiency among ESCs derived from IVF and *in vivo* embryos (Horii, Yanagisawa et al. 2010). However, DFS-ICSI is a more invasive technique that bypasses the natural selection barriers allowing damaged sperm to fertilize the oocyte (Schultz and Williams 2002, Hourcade, Perez-Crespo et al. 2010). In fact, low rates of successful implantation and fetal development have been described for DFS-ICSI-generated embryos (Moreira, Perez-Crespo et al. 2007). Both ESCs derivation and implantation failures could be explained by karyotype anomalies, as 40% of embryos generated by ICSI using DFS show abnormal chromosome segregation and chromosome fragmentation (Yamagata, Suetsugu et al. 2009).

Consequently, embryonic characteristics and alterations are reflected in their corresponding ESCs lines; however, continuous passaging directs them to adopt similar profiles.

Embryonic characteristics can be reflected in other cell lines as well. Intriguingly, in chapter IV, after developing a system to efficiently establish trophectoderm cell lines from bovine embryo biopsies, high variability in gene expression at early passages was observed among cell lines obtained under the same derivation conditions, so differences should be due to the embryonic source. This finding is consistent with a previous study

that derived 12 bovine trophoblastic cell lines and quantified the expression of trophoblastic genes: placental lactogen (*CSH1*), interferon-*t (IFNt)*, pregnancy-associated glycoprotein 1 (*PAG1*), and prolactin-related protein 1 (*PRP1*); and found different expression patterns among the cell lines with no correlations with culture conditions (Suzuki, Koshi et al. 2011). Thus, in this chapter IV it was demonstrated that embryonic characteristics influence trophoblastic cell lines derivation as well.

Culture conditions affect cell lines derivation

Culture conditions are a main factor affecting cell lines derivation and characteristics. In this thesis, the effect of culture conditions has been studied in ESCs lines in chapter II, and in trophoblastic cell lines in chapter IV.

In chapter II, the effect of leukemia inhibitory factor (LIF) supplementation during preimplantational embryo culture up to blastocyst for ESCs derivation was analyzed due to the contradictory evidences existing among previous studies (Jurisicova, Ben-Chetrit et al. 1995, Dunglison, Barlow et al. 1996, Tsai, Chang et al. 2000, Rungsiwiwut, Rungarunlert et al. 2008). Although the embryos supplemented with LIF showed a lower total cell number, the ratio of inner cell mass (ICM)/total cells was significantly higher. Furthermore, these embryos proved to be more suitable for ESCs isolation, as their derivation efficiency was higher. Additionally, regular medium for ESCs lines derivation was supplemented with growth factors regularly used for germline stem cells (GSCs) culture (EGF, GDNF and bFGF), and ESCs derivation efficiency increased in the same way. In agreement to this observation, the beneficial effect of these factors on other pluripotent cells populations has been previously reported (Reynolds and Weiss 1992, Donovan and de Miguel 2003, Kanatsu-Shinohara, Ogonuki et al. 2003).

The vast effect of culture medium supplementation with different factors has been exhaustively studied in ESCs. In chapter II, ESCs were derived over a feeder layer of mouse embryonic fibroblasts (EFs) in medium supplemented with: fetal calf serum (FCS) and LIF (ES medium); FCS, LIF and GSCs-related growth factors (GS medium); or FCS, LIF and inhibitory factors (2i medium). We could observe an improvement in ESCs derivation efficiency in GS medium compared to ES medium, and ESCs lines derived in GS medium fulfilled pluripotency criteria and showed a higher chimeric mice generation ability. Thus, a possible synergy among LIF supplementation during embryo culture and the posterior addition of growth factors present in GS medium that favours ESCs derivation. Different culture systems have been established to avoid the variability induced by FCS, which shows variations between batches, as knockout serum replacement (KSR) (Cheng, Dutra et al. 2004) and small-molecule inhibitors (MEK and GSK3*β*; 2i) (Ying, Wray et al. 2008). The 2i culture system allows efficient derivation and expansion of germline-competent

ESCs from different strains of mice and species that were previously recalcitrant for ESCs derivation (Buehr, Meek et al. 2008, Nichols, Jones et al. 2009). Thus, ESCs derivation efficiency is highly dependent on culture medium. In the same way, transcriptome is defined by culture conditions. In chapter II, gene expression in ESCs lines varied according to the different culture media employed for their derivation (ES, GS and 2i). Indeed, it has been reported that culture conditions are the major aspect determining gene expression, over embryonic origin and derivation procedure (Marks, Kalkan et al. 2012, Leitch, McEwen et al. 2013).

In chapter IV, different media were used with the aim of improving derivation efficiency of trophoblastic cell lines. Although we found that a derivation procedure allowing a rapid adhesion of the embryo or biopsy to the culture plate was a critical step and significantly increased derivation efficiency, culture medium was decisive as well. Conditioned culture medium contains secreted proteins that include numerous enzymes, growth factors, cytokines and hormones (Dowling and Clynes 2011). Most of the studies regarding trophoblast cell lines derivation employed conditioned medium by co-cultivation with mouse embryonic fibroblasts (Talbot, Caperna et al. 2000, Talbot, Caperna et al. 2004, Talbot, Powell et al. 2007). However, co-cultivation has the disadvantage of contaminating the cell lines of interest, so in our study trophoblastic cells were cultured over a gelatinized culture plate, and different conditioned media from mouse embryonic fibroblasts (Cm), bovine embryonic fibroblasts (Cb) and bovine oviductal cells (Co) were assayed. Except for Cb, conditioned media improved derivation efficiency, being Cm the most efficient medium for trophoblastic cell lines derivation. Other articles previously used bovine endometrial fibroblast-conditioned medium to derive trophoblastic cell lines (Shimada, Nakano et al. 2001, Hashizume, Shimada et al. 2006); however, heterologous conditioned medium by mouse embryonic fibroblasts was the most efficient for rapid adhesion and cell growth in our study. Furthermore, conditioned media were supplemented with EGF, since earlier studies demonstrated its positive effect on trophoblastic cells proliferation (Hambruch, Haeger et al. 2010). In conclusion, our observations and those from other authors indicate that trophoblastic cell lines derivation efficiency significantly depends on culture conditions.

A germ cell origin for pluripotent cells

During the transition from blastocyst cells to pluripotent ESCs, the developmental program in ICM cells is modified in order to acquire the ability to divide indefinitely while maintaining the potential to differentiate towards any tissue. Several evidences such as similarities existing among ESCs and EGCs (Matsui, Zsebo et al. 1992, Resnick, Bixler et al. 1992), expression of key pluripotency genes *Pou5f1, Sox2* and *Nanog* in PGCs (Kehler,

Tolkunova et al. 2004, Yamaguchi, Kimura et al. 2005, Chambers, Silva et al. 2007, Durcova-Hills and Surani 2008, Chu, Surani et al. 2011, Zhao, Ji et al. 2012), or the fact that germ-cell markers as *Blimp1*, *Dppa3*, *Ifitm3*, *Piwil2*, *Dazl* or *Ddx4* are expressed in pluripotent cells (Geijsen, Horoschak et al. 2004, Qing, Shi et al. 2007, Mise, Fuchikami et al. 2008, Xu, Pantakani et al. 2011) have led to the thought that all pluripotent cells may have a common germ cell origin (Zwaka and Thomson 2005).

In chapter II, blastocysts were cultured in a germ cell-specific medium (GS) to favor ESCs lines derivation through this germ cell-like state, and efficiency was significantly higher in GS medium than in regular ES medium. Germ cell-specific genes were expressed in ESCs derived in ES and GS culture conditions, suggesting that GS medium did not induce a significantly higher swift towards germ cell specification. In contrast, upregulation of *Stella* and downregulation of several germline markers was observed in 2i conditions, indicating a different transitional state from ICM cell to ESC, or suggesting the existence of a different proportion among the ICM-like or epiblst and germline-like subpopulations during the process.

The appearance of this germ cell-like intermediate state has been reported in other articles that used standard FCS culture conditions for ESCs derivation (Tang, Barbacioru et al. 2010), but it seems to be facultative for the stabilization of pluripotency *in vitro,* since it has been described that culture in 2i conditions enables the effective direct recruitment of ESCs skipping this state (Chu, Surani et al. 2011). 2i conditions employed by these authors (Nichols, Silva et al. 2009, Chu, Surani et al. 2011) consisted of a gelatinized culture plate without mouse EF and N2B27 medium supplemented with LIF and the two inhibitors, avoiding the use of FCS. Consequently, it could be possible that the intermediate germ cell-like state is induced by FCS or by other factors secreted by mouse EF.

However, another study demonstrated that this germ cell-like state can be absent under LIF and FCS conditions when epiblast stem cells (EpiSCs) are reverted to ESCs lacking the main regulator of PGCs specification *Blimp1* (Bao, Leitch et al. 2012), though this is a different model. Nevertheless, most of the studies regarding the germ cell-like intermediate state during ESCs derivation are mainly based on *Blimp1* presence or absence. However, although *Blimp1* is a master regulator for PGCs specification, other genes play important roles as well, and they should be taken into consideration. In this regard, *Prdm14*, together with *Blimp1*, plays a critical role in early germ cells specification, epigenetic reprogramming and re-expression of pluripotency genes (Ohinata, Payer et al. 2005, Yamaji, Seki et al. 2008). Another crucial factor for PGCs development, *Prmt5*, together with *Blimp1* and *Prdm14*, has the potential to reprogram somatic cells into induced pluripotent stem cells (iPSCs) (Nagamatsu, Kosaka et al. 2011). Other authors

demonstrated that *Prdm14* accelerates the EpiSCs reprogramming process to ESCs when expressed in combination with *Klf2* in classical culture conditions with FCS, and induces the expression of several germline-associated genes as *Blimp1*, *Dppa3*, *Ifitm3* and *Nanos3*, indicating that the reprogramming process may require progression through the germ cell-like intermediate. However, reprogramming was not affected in the absence of *Blimp1* (Gillich, Bao et al. 2012). Thus, it is possible that *Prdm14* and *Blimp1* have redundant activities during the establishment of the pluripotent state (Geijsen 2012). *Prdm14* also ensures naïve pluripotency in ESCs in 2i conditions by antagonizing FGF receptor signaling and by repressing expression of *de novo* DNA methyltransferases that modify the epigenome to a primed state (Yamaji, Ueda et al. 2013).

Studies reporting that germline, premeiotic or meiotic markers such as *Blimp1*, *Dppa3*, *Ifitm3*, *Piwil2*, *Dazl*, *Ddx4*, *Stra8*, *Rnf17*, *Rnh2*, *Sycp3*, *Pgk2* or *Creb3/4* are expressed in ESCs or in other pluripotent populations as iPSCs, GSCs and EGCs, were performed under classical culture conditions over a mouse EF feeder layer and medium supplemented with LIF and FCS (Geijsen, Horoschak et al. 2004, Qing, Shi et al. 2007, Mise, Fuchikami et al. 2008, Tang, Barbacioru et al. 2010, Xu, Pantakani et al. 2011). Consequently, according to our results in chapter II and to those reported by other authors, it seems that during ESCs derivation, cells undergo a germ cell-like intermediate state under commonly used culture conditions, although pluripotency can be directly captured from the epiblast by MEK and GSK3β inhibitors supplementation without FCS and EF feeder layer.

Thus, ESCs in serum conditions consist of a mixed population of cells with naïve ICM-like properties and cells with primed epiblast or germline-like properties, and these two populations would interchange their identities during culture maintaining a dynamic equilibrium, where *Prdm14* is associated with the naïve ICM-like properties (Yamaji, Ueda et al. 2013). By the contrary, ESCs in 2i conditions appear to be homogeneous and have a gene expression profile and epigenome different to ESCs cultured in serum conditions and more similar to those of ICM cells (Ying, Wray et al. 2008, Marks, Kalkan et al. 2012). Our results also show that ES and GS media produce a higher expression of some germinal markers during the transition from blastocyst embryonary cells to ESCs, while 2i medium produces an upregulation of *Stella*, suggesting that it could exist a different proportion of the subpopulations present in ESCs, prevailing the epiblast or germline-like subpopulation in ES or GS culture conditions, while the ICM-like subpopulation would prevail in 2i conditions. It has been described that culture conditions that preserve ESCs pluripotency would maintain a balanced proportion among both subpopulations (Hayashi, Lopes et al. 2008).

In vitro cultured pluripotent cells are not the only ones that seem to retain a germinal origin, as germline-specific genes have been localized *in vivo* in different tissues holding

multipotent cells populations. In chapter III, we studied germline-specific gene *Dazl* throughout development by a *Dazl*-eGFP-transgenic mouse. Preimplantational embryos, foetal, neonatal and adult tissues were analyzed for *Dazl*-driven-eGFP expression. During preimplantational embryo development, *Dazl*-eGFP was detected from zygote to blastocysts. According to our results, *zDazl* has been localized in early zebrafish embryos (Maegawa, Yasuda et al. 1999, Hashimoto, Maegawa et al. 2004) and *XDazl* in Xenopus embryos until stage 10 (Houston and King 2000). In the same way, *Dazl* (Pan, Liao et al. 2008, Chen, Melton et al. 2011) and other germline-related genes such as *Dppa3* and *Ddx4* (Sato, Kimura et al. 2002, Xu, Pantakani et al. 2011) have been localized from zygote to blastocyst stages in mouse.

Dazl-eGFP was localized during fetal development and adulthood in other tissues besides gonads as intestine and bone marrow. Interestingly, these tissues contain different adult multipotent cells populations, such as intestinal stem cells (Barker, van Es et al. 2007, van der Flier and Clevers 2009) and bone marrow mesenchymal stem cells (Gnecchi and Melo 2009). According to our results, other studies have reported the expression of germline-specific genes as *Pou5f1*, *Dppa3*, *Ifitm3*, *Dazl* and *Ddx4* in mouse and human bone marrow (Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Katsara, Mahaira et al. 2011). Furthermore, *DAZL* has been located in human amniotic fluid cells (Stefanidis, Loutradis et al. 2008, Stefanidis, Pergialiotis et al. 2013). It has been hypothesized that a population of very small embryonic stem cells (VSELs) reside in murine bone marrow and other adult organs that express several germline-specific markers and play an important role in the turnover of tissue-specific multipotent cells. These cells would originate from the germline and would be deposited during early gastrulation in developing tissues and organs (Ratajczak, Shin et al. 2010).In conclusion, besides the largely known function of *Dazl* in germ cell development, *Dazl*-eGFP and other germline-specific genes expression in other tissues harboring pluripotent cells may suggest that adult stem cells share a common germ cell-like origin with other *in vitro* cultured pluripotent populations.

Presence of multipotent cells in adult tissues and in bovine trophoblast cell lines

The tissues exhibiting a high cell turnover as intestine, skin, skeletal muscle or bone marrow, are supported by multipotent cells that are able to self-renew for long periods and to differentiate towards a specific cell lineage (Macaluso and Myburgh 2012). Some of these cells have been identified through the expression of certain pluripotency markers as *Oct4*, *Sox2*, *Nanog*, *Klf4*, *Lin28* or *SSEA1*. However, other markers are specific for every multipotent cells population; for instance, *Lgr5* for intestinal stem cells (van der Flier and Clevers 2009); CD48, CD150 or CD244 for hematopoietic stem cells (Kim, He et al. 2006);

CD133, CD15 or CD24 for neural stem cells (Rietze, Valcanis et al. 2001, Capela and Temple 2002, Corti, Nizzardo et al. 2007, Peh, Lang et al. 2009); and CD10, CD13, CD73, CD105 or CD271 for mesenchymal stem cells (Mafi, Hindocha et al. 2011).

Furthermore, some germline-specific markers such as *Dppa3*, *Ifitm3*, *Dazl* and *Ddx4* have been localized in tissues harboring multipotent cells (Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Stefanidis, Loutradis et al. 2008, Katsara, Mahaira et al. 2011, Stefanidis, Pergialiotis et al. 2013). Similarly, in chapter III *Dazl*-eGFP expression was found in bone marrow and intestine, probably due to the presence of hematopoietic and mesenchymal stem cells residing in bone marrow, and to intestinal stem cells located in the crypts of Lieberhahn (Schabort, Myburgh et al. 2009). Therefore, *Dazl*-GFP transgene could be used to explore the presence of multipotent cells in different tissues.

Trophoblast stem cells (TSCs) represent another multipotent cells population residing in the trophectoderm (TE) (Tanaka, Kunath et al. 1998). However, TSCs have been isolated and well characterized only in mouse and rhesus macaque (Vandevoort, Thirkill et al. 2007, Roberts and Fisher 2011). In contrast, the existence and location of a TSCs niche in domestic species remains unknown, in part due to a different TE development (Bindon 1971, Geisert, Brookbank et al. 1982, Thatcher, Meyer et al. 1995). Consequently, TSCs have not been isolated in these species (Roberts and Fisher 2011); although primary trophectoderm cell lines have been derived from sheep and goat (Miyazaki, Imai et al. 2002), pig (Ramsoondar, Christopherson et al. 1993, Flechon, Laurie et al. 1995, La Bonnardiere, Flechon et al. 2002) and cattle (Talbot, Caperna et al. 2000, Shimada, Nakano et al. 2001).

Interestingly, bovine trophoblast cell lines obtained in chapter IV showed a long-term steady cell proliferation *in vitro*, and expressed several pluripotency-related genes as *POU5F1, CDH1, FGF4, TERT*, and *TERC*. According to our results, other studies have suggested that trophoblastic cells are not terminal cells and present some pluripotency features (Suzuki, Koshi et al. 2011, Schiffmacher and Keefer 2013). Thus, a population of multipotent cells could be present also in bovine trophoblastic cell lines.

Discusión general

En las últimas décadas se han alcanzado grandes avances en investigación con células pluripotentes; sin embargo, todavía se desconocen las condiciones óptimas para su identificación, aislamiento y cultivo *in vitro*. Por ello, en esta tesis se han querido abarcar varios aspectos determinantes para la obtención de líneas celulares, como el efecto de la fuente embrionaria o de las condiciones de cultivo. Además se ha establecido un sistema para obtener líneas celulares trofoblásticas a partir de biopsias embrionarias bovinas, que permita llevar a cabo estudios de placentación y diagnóstico genético y epigenético preimplantacional.

La fuente embrionaria afecta a la obtención de líneas de células pluripotentes

Las ESCs han sido las células pluripotentes más comúnmente utilizadas desde su aislamiento hace más de tres décadas (Evans and Kaufman 1981). Las técnicas de aislamiento de ESCs y los sistemas de cultivo han evolucionado en los últimos años para mejorar la eficiencia de obtención, pero existen discrepancias acerca de si las características embrionarias influyen o no en el proceso.

Las técnicas de reproducción asistida como el cultivo *in vitro* embrionario, la fecundación *in vitro* (IVF), la inyección intracitoplasmática de espermatozoides (ICSI) o la transferencia nuclear de células somáticas (SCNT) han contribuido al desarrollo de tratamientos de infertilidad en humanos y de nuevos métodos de reproducción en animales. Sin embargo, la manipulación y el cultivo *in vitro* de los embriones se han asociado con la aparición de alteraciones genéticas, epigenéticas y en la ultraestructura de los embriones, y con síndromes y enfermedades durante la edad adulta (Rizos, Fair et al. 2002, Corcoran, Fair et al. 2006, Fernandez-Gonzalez, Moreira et al. 2008, Calle, Miranda et al. 2012). La clasificación de los embriones en función de su calidad tras llevar a cabo técnicas de reproducción asistida está basada en gran medida en criterios subjetivos. Además, en muchas ocasiones se pasa por alto que las ESCs humanas proceden de embriones producidos *in vitro*, muchas veces sobrantes o descartados por no ser válidos para su transferencia en las clínicas de infertilidad. Estas circunstancias hacen cuestionarse si las ESCs retienen cierta memoria de los embriones de los que proceden.

En el capítulo I llevamos a cabo la técnica de ICSI con espermatozoides con DNA fragmentado (DFS), modelo previamente desarrollado en el laboratorio (Fernandez-Gonzalez, Moreira et al. 2008) que produce embriones de “mala calidad”, y pudimos demostrar que estos embriones tienen un menor potencial para generar líneas de ESCs que los embriones producidos *in vivo*. Asimismo, en pases tempranos estas ESCs producidas por ICSI con DFS difirieron de las ESCs procedentes de embriones *in vivo* en la expresión de ciertos genes relacionados con la pluripotencia y la represión epigenética, el daño y la reparación del ADN, y de ADN metil-transferasas *de novo* e histona-deacetilasas.

Sin embargo, en pases tardíos no se observaron diferencias en la expresión génica. En la misma línea de nuestros resultados, un estudio previo describió que las ESCs retienen diferencias epigenéticas en función del origen *in vivo* o producido por IVF del embrión del que proceden, y que estas diferencias desaparecen tras varios pases celulares (Horii, Yanagisawa et al. 2010). Otro estudio en el que se describieron las diferencias a nivel transcripcional entre las ESCs obtenidas a partir de embriones cultivados *in vitro* e *in vivo* sugirió que estas diferencias se mantienen estables durante el cultivo *in vitro* a largo plazo (Harvey, Mao et al. 2012). No obstante, la teoría de que las células pluripotentes de diferentes orígenes pueden adoptar perfiles genéticos y epigenéticos similares tras varios pases celulares se ha demostrado previamente en ESCs (Ramirez, Pericuesta et al. 2007, Horii, Yanagisawa et al. 2010) y en iPSCs (Polo, Liu et al. 2010).

Las diferencias en la expresión génica observadas en pases tempranos en las ESCs producidas por ICSI con DFS podrían reflejar las alteraciones que han sido previamente descritas en embriones y en animales adultos (Fernandez-Gonzalez, Moreira et al. 2008). Las alteraciones epigenéticas descritas en los embriones producidos por ICSI con DFS podrían estar relacionadas con la represión de la expresión de la histona-deacetilasa *Hdac10* y de las ADN metil-transferasas *de novo Dnmt3a* y *Dnmt3b* observada en las líneas de ESCs procedentes de ICSI con DFS. Recientemente se ha descrito un *splicing* alternativo de *Dnmt3b* que carece del exón 6 (*Dnmt3bΔ6*), que muestra una menor actividad que la forma original de *Dnmt3b,* e induce un estado de hipometilación. *Dnmt3bΔ6* se expresa específicamente en embriones de ratón procedentes de cultivo *in vitro* y se transmite a las ESCs obtenidas a partir de ellos (Horii, Suetake et al. 2011). El envejecimiento prematuro observado en los animales producidos por ICSI con DFS podría estar relacionada con la baja expresión de la enzima reparadora *Alkbh3* (Morita, Nakane et al. 2010) y con la alta expresión de *Gadd45* (Moskalev, Smit-McBride et al. 2012) observadas en las líneas ESCs procedentes de ICSI con DFS.

Por otra parte también se vio afectada la pluripotencia de las líneas de ESCs producidas por ICSI con DFS, ya que se estas líneas mostraron una menor expresión de *Sox2* y una reducida eficiencia de obtención. Por el contrario, Horii *et al.* no encontraron diferencias en la eficiencia de obtención entre ESCs obtenidas a partir de embriones producidos *in vivo* o mediante IVF (Horii, Yanagisawa et al. 2010). Sin embargo, la ICSI con DFS es una técnica más invasiva que elude las barreras naturales de selección espermática, permitiendo que espermatozoides dañados fertilicen el ovocito (Schultz and Williams 2002, Hourcade, Perez-Crespo et al. 2010). De hecho, se ha descrito que estos embriones muestran bajos índices de implantación y de desarrollo fetal (Moreira, Perez-Crespo et al. 2007). Tanto la baja eficiencia de obtención de ESCs como los fallos de implantación de los embriones podrían deberse a anomalías en el cariotipo, ya que el 40% de los

embriones producidos por ICSI con DFS muestran fragmentación cromosómica y una segregación cromosómica anormal (Yamagata, Suetsugu et al. 2009).

Por lo tanto, las características o alteraciones embrionarias se ven reflejadas en sus correspondientes líneas de ESCs; sin embargo, el cultivo a largo plazo hace que adopten perfiles similares.

Las características embrionarias podrían verse reflejadas también en otras líneas celulares. Curiosamente, en el capítulo IV, tras desarrollar un sistema para establecer de manera eficiente líneas de células trofoblásticas bovinas a partir de biopsias embrionarias, pudimos observar una gran variabilidad en la expresión génica en pases tempranos de líneas celulares procedentes de las mismas condiciones de obtención, por lo que las diferencias podrían tener su origen en la fuente embrionaria. Este hallazgo se corresponde con lo observado en un estudio previo en el que se obtuvieron 12 líneas de células de trofoblásticas bovinas y se cuantificó la expresión de los genes específicos de células trofoblásticas: interferón tau (*IFNt*), lactógeno placentario (*CSH1*), proteína relacionada con la prolactina 1 (*PRP1*) y glicoproteína asociada a la gestación (*PAG1*); encontrando diferentes patrones de expresión entre las líneas celulares sin relación alguna con las condiciones de cultivo (Suzuki, Koshi et al. 2011). Por lo tanto, en el capítulo IV se demostró que las características embrionarias afectan también a la obtención de líneas de células trofoblásticas bovinas.

Las condiciones de cultivo afectan a la obtención de líneas celulares

Las condiciones de cultivo son un factor fundamental que afecta a la obtención de líneas celulares y a sus características. En esta tesis, el efecto de las condiciones de cultivo se ha analizado en líneas de ESCs en el capítulo II, y en líneas de células trofoblásticas en el capítulo IV.

En el capítulo II, se analizó el efecto de la adición de “leukemia inhibitory factor” (LIF) al medio durante el cultivo del embrión preimplantacional hasta el estadio de blastocisto para su posterior uso en la obtención de ESCs, debido a la información contradictoria procedente de estudios previos (Jurisicova, Ben-Chetrit et al. 1995, Dunglison, Barlow et al. 1996, Tsai, Chang et al. 2000, Rungsiwiwut, Rungarunlert et al. 2008). A pesar de que los embriones cultivados con LIF mostraron un menor número total de células, el índice de células de la masa celular interna (ICM)/células totales fue significativamente mayor en estos embriones. Además, resultaron ser más aptos para el aislamiento de ESCs, ya que mostraron una mayor eficiencia de obtención. Asimismo se suplementó el medio tradicional para el cultivo de ESCs con factores de cultivo normalmente usados para el cultivo de células germinales embrionarias (GSCs) (EGF, GDNF y bFGF), y la eficiencia de

obtención de ESCs también se vio incrementada. En concordancia con nuestro estudio, el efecto beneficioso de estos factores en otras poblaciones de células pluripotentes ha sido previamente demostrado (Reynolds and Weiss 1992, Donovan and de Miguel 2003, Kanatsu-Shinohara, Ogonuki et al. 2003).

El enorme efecto de suplementar el medio de cultivo con diferentes factores ha sido ampliamente estudiado en las ESCs. En el capítulo II, las ESCs fueron obtenidas sobre una monocapa de fibroblastos embrionarios de ratón en diferentes medios suplementados con: suero fetal bovino (FCS) y LIF (medio ES); FCS, LIF y factores de crecimiento de GSCs (medio GS); o FCS, LIF y factores inhibidores (medio 2i). Se observó un incremento en la eficiencia de obtención de ESCs en el medio GS en comparación con el medio ES, y las líneas de ESCs obtenidas en medio GS cumplieron los criterios de pluripotencia y mostraron una mayor capacidad de formación de ratones quiméricos. Se manifiesta por tanto una posible sinergia entre la suplementación de LIF durante el cultivo embrionario *in vitro* y la adición posterior de otros factores de crecimiento presentes en el medio GS, que favorece la obtención de nuevas líneas de ESCs. Diferentes sistemas de cultivo como el "knockout serum replacement" (KSR) (Cheng, Dutra et al. 2004) y los inhibidores (MEK y GSK3*β*; 2i) han sido desarrollados para evitar la variabilidad inducida por el uso de FCS, ya que existen grandes variaciones entre lotes (Ying, Wray et al. 2008). El sistema 2i permite la eficiente obtención y expansión de ESCs capaces de contribuir al linaje germinal en animales quiméricos en diferentes cepas de ratones y en especies en las que previamente no había sido posible (Buehr, Meek et al. 2008, Nichols, Jones et al. 2009). Por lo tanto, la obtención de ESCs depende enormemente del medio de cultivo. Del mismo modo, el transcriptoma está definido fundamentalmente por las condiciones de cultivo. En el capítulo II, la expresión génica de las líneas de ESCs fue diferente en función del medio de cultivo utilizado para su obtención (ES, GS y 2i). De hecho, se ha descrito que las condiciones de cultivo son el aspecto que más condiciona la expresión génica, por encima del origen embrionario y del procedimiento de obtención de las ESCs (Marks, Kalkan et al. 2012, Leitch, McEwen et al. 2013).

En el capítulo IV se utilizaron diferentes medios de cultivo con el objetivo de mejorar la eficiencia de obtención de líneas de células trofoblásticas bovinas. A pesar de que observamos que una rápida adhesión del embrión o biopsia a la placa de cultivo es primordial, e incrementa significativamente la eficiencia de obtención de líneas celulares, el medio de cultivo es decisivo también. Los medios de cultivo condicionados contienen diferentes proteínas secretadas que incluyen enzimas, factores de crecimiento, citoquinas y hormonas (Dowling and Clynes 2011). En muchos de los estudios en los que se obtuvieron líneas de células trofoblásticas bovinas se empleó medio condicionado al llevar a cabo un co-cultivo con fibroblastos embrionarios murinos (Talbot, Caperna et al. 2000, Talbot, Caperna et al. 2004, Talbot, Powell et al. 2007). Sin embargo, el co-cultivo

tiene como inconveniente la contaminación de las líneas celulares de interés con otro tipo celular, por lo que en nuestro trabajo las células trofoblásticas fueron cultivadas en un sistema en microgota gelatinizada, utilizando diferentes medios de cultivo condicionados por fibroblastos embrionarios murinos (Cm), fibroblastos embrionarios bovinos (Cb) y células oviductales ovinas (Co). A excepción del medio Cb, los medios condicionados mejoraron la eficiencia, siendo el medio Cm el más apropiado para la obtención de líneas de células trofoblásticas. En otros artículos anteriores se había usado medio condicionado por fibroblastos endometriales bovinos para obtener líneas de células trofoblásticas (Shimada, Nakano et al. 2001, Hashizume, Shimada et al. 2006); sin embargo nuestro medio de cultivo heterólogo condicionado por fibroblastos embrionarios murinos fue el más eficaz, permitiendo una rápida adhesión del embrión o biopsia, y el posterior crecimiento celular. Además, los medios de cultivo fueron suplementados con EGF, ya que estudios anteriores habían demostrado su efecto positivo en la proliferación de células trofoblásticas (Hambruch, Haeger et al. 2010). En conclusión, nuestras observaciones junto con las de otros autores indican que la eficiencia de obtención de líneas de células trofoblásticas bovinas depende en gran medida de las condiciones de cultivo.

Un origen germinal para las células pluripotentes

Diversas evidencias como la similitud existente entre las ESCs y las EGCs (Matsui, Zsebo et al. 1992, Resnick, Bixler et al. 1992), la expresión de diversos genes de pluripotencia como *Pou5f1*, *Sox2* y *Nanog* en células primordiales germinales (PGCs) (Kehler, Tolkunova et al. 2004, Yamaguchi, Kimura et al. 2005, Chambers, Silva et al. 2007, Durcova-Hills and Surani 2008, Chu, Surani et al. 2011, Zhao, Ji et al. 2012), o el hecho de que marcadores específicos de linaje germinal como *Blimp1*, *Dppa3*, *Ifitm3*, *Piwil2*, *Dazl* o *Ddx4* se expresen en células pluripotentes (Geijsen, Horoschak et al. 2004, Qing, Shi et al. 2007, Mise, Fuchikami et al. 2008, Xu, Pantakani et al. 2011) han llevado a pensar que todas las células pluripotentes podrían tener un origen germinal común (Zwaka and Thomson 2005).

En el capítulo II se cultivaron blastocistos en un medio específico para células germinales (GS) para favorecer la obtención de líneas de ESCs a través de este posible estado transitorio similar a una célula germinal, y la eficiencia de obtención fue significativamente mayor en el medio GS que en el medio ES. Sin embargo, no pudimos encontrar indicios de una diferenciación más pronunciada hacia el linaje germinal inducida por el medio GS, ya que encontramos expresión de genes específicos de linaje germinal en todas las condiciones de cultivo (GS, ES y 2i), lo que apunta a la aparición de este estado similar a una célula germinal durante el proceso de obtención de ESCs.

La aparición de este intermediario común se ha descrito en otros artículos que utilizaron condiciones de cultivo estándar con FCS para la obtención de ESCs (Tang, Barbacioru et al. 2010), pero parece no ser estrictamente necesario para la estabilización de la pluripotencia *in vitro*, ya que el cultivo en condiciones 2i permite el reclutamiento directo de ESCs de manera efectiva sorteando este estado similar a una célula germinal (Chu, Surani et al. 2011). En nuestro estudio, los inhibidores de MEK y GSK3β fueron usados junto con FCS y LIF sobre una monocapa de EF de ratón. Por el contrario, las condiciones de cultivo 2i utilizadas por otros autores (Nichols, Silva et al. 2009, Chu, Surani et al. 2011) consistieron en una placa de cultivo gelatinizada sin EF murinos y medio N2B27 suplementado con LIF y los inhibidores de MEK y GSK3β, evitando el uso de FCS. Consecuentemente, es posible que el estado intermedio similar a una célula germinal esté inducido por el FCS o por otros factores secretados por los EF murinos.

Sin embargo, un estudio ha demostrado que este estado similar a una célula germinal puede estar ausente también en condiciones de cultivo con FCS y LIF cuando células troncales del epiblasto (EpiSCs) carentes del principal regulador de la especificación de las PGCs, *Blimp1,* son revertidas a ESCs (Bao, Leitch et al. 2012). No obstante, la mayoría de los autores que han investigado la existencia de este estado similar a una célula germinal durante la obtención de ESCs se han basado principalmente en la presencia o ausencia de *Blimp1*. Es cierto que *Blimp1* es fundamental durante la especificación de las PGCs, pero existen otros genes que también juegan papeles elementales y deberían ser analizados igualmente. De hecho, *Prdm14*, junto con *Blimp1*, juega un papel esencial en la especificación temprana de las células germinales (Ohinata, Payer et al. 2005, Yamaji, Seki et al. 2008). Otro factor fundamental para el desarrollo de las PGCs, *Prmt5*, junto con *Blimp1* y *Prdm14*, tiene el potencial de reprogramar células somáticas en iPSCs. (Nagamatsu, Kosaka et al. 2011). Otros autores han demostrado que *Prdm14* acelera la reprogramación de EpiSCs hacia ESCs cuando se expresa en combinación con *Klf2* en condiciones de cultivo con FCS, e induce la expresión de diversos genes asociados con el linaje germinal como *Blimp1*, *Dppa3*, *Ifitm3* y *Nanos3*, indicando que este proceso de reprogramación también podría requerir un paso intermedio similar a una célula germinal. Sin embargo, la ausencia de *Blimp1* no afecta a dicho proceso de reprogramación (Gillich, Bao et al. 2012). Por ello, es posible que *Prdm14* y *Blimp1* tengan una actividad redundante durante el establecimiento de la pluripotencia (Geijsen 2012). *Prdm14* también garantiza el estado de pluripotencia naïve en ESCs al antagonizar la señalización del receptor de FGF y reprimir la expresión de ADN metil-transferasas *de novo* que modifican el epigenoma hacia un estado *primed* (Yamaji, Ueda et al. 2013).

Los estudios que muestran que los marcadores de linaje germinal, premeióticos o meióticos como *Blimp1*, *Dppa3*, *Ifitm3*, *Piwil2*, *Dazl*, *Ddx4*, *Stra8*, *Rnf17*, *Rnh2*, *Sycp3*, *Pgk2* o *Creb3/4* se expresan en ESCs o en otras poblaciones de células pluripotentes como las

iPSCs, las GSCs o las EGCs, se han llevado a cabo en condiciones clásicas de cultivo con FCS (Geijsen, Horoschak et al. 2004, Qing, Shi et al. 2007, Mise, Fuchikami et al. 2008, Tang, Barbacioru et al. 2010, Xu, Pantakani et al. 2011). Consecuentemente, de acuerdo con los resultados descritos en el capítulo II de esta tesis y con aquellos descritos por otros autores, parece que durante la obtención de ESCs bajo ciertas condiciones de cultivo, las células pasan por un estado intermedio similar a una célula germinal, aunque la pluripotencia puede ser capturada directamente del epiblasto gracias a los inhibidores de MEK and GSK3β pero sin utilizar FCS ni una monocapa de fibroblastos murinos.

Por lo tanto, las ESCs cultivadas con FCS están constituidas por una población mixta de células con propiedades naïve similares a las células de la ICM, y células con propiedades *primed* similares a las del epiblasto; y ambas poblaciones intercambiarían sus identidades durante el cultivo manteniendo un equilibrio dinámico, estando *Prdm14* asociado con las propiedades naïve de la ICM (Yamaji, Ueda et al. 2013). Por el contrario, las ESCs en condiciones 2i parecen ser una población homogénea que tiene un perfil de expresión génica y un epigenoma diferente a los de las ESCs cultivadas con FCS y similar a los de las células de la ICM (Ying, Wray et al. 2008, Marks, Kalkan et al. 2012). Nuestros resultados muestran además cómo los medios ES y GS producen una mayor expresión de algunos marcadores germinales durante la transición de las células embrionarias del blastocisto a ESCs, mientras que el medio 2i produce un aumento de *Stella*, sugiriendo que existe una distinta proporción de las subpoblaciones presentes en las ESCs, siendo predominante la subpoblación similar al epiblasto o a la línea germinal en las condiciones de cultivo ES o GS, mientras que en condiciones 2i sería predominante la subpoblación similar a la ICM. Se ha descrito que durante el cultivo en condiciones que mantienen la pluripotencia de las ESCs, las proporciones ambas subpoblaciones permanecen en equilibrio (Hayashi, Lopes et al. 2008).

No sólo las células pluripotentes cultivadas *in vitro* parecen tener un origen germinal, ya que también se han localizado genes específicos de linaje germinal *in vivo* en diferentes tejidos que albergan poblaciones de células pluripotentes. En el capítulo III se ha estudiado el gen específico de linaje germinal *Dazl* a lo largo del desarrollo mediante un ratón transgénico *Dazl*-eGFP, en el que se analizó la expresión de eGFP inducida por *Dazl* en el desarrollo embrionario y en los tejidos fetales, neonatales y adultos. Durante el desarrollo preimplantacional embrionario, la expresión de *Dazl*-eGFP fue detectada desde el estadio de cigoto hasta el de blastocisto. En consonancia con nuestros resultados, se ha descrito la expresión de *zDazl* en embriones tempranos de pez cebra (Maegawa, Yasuda et al. 1999, Hashimoto, Maegawa et al. 2004) y de *XDazl* en embriones de Xenopus hasta el estadio 10 (Houston and King 2000). Del mismo modo, en el ratón se ha localizado *Dazl*

entre los estadios de cigoto y blastocisto (Pan, Liao et al. 2008, Chen, Melton et al. 2011), así como otros genes relacionados con el linaje germinal, como *Dppa3* y *Ddx4* (Sato, Kimura et al. 2002, Xu, Pantakani et al. 2011).

La expresión de *Dazl*-eGFP fue encontrada durante el desarrollo fetal y la edad adulta en otros tejidos además de las gónadas, como son el intestino y la médula ósea. Curiosamente, en estos tejidos residen diferentes poblaciones de células pluripotentes adultas como las células troncales intestinales (Barker, van Es et al. 2007, van der Flier and Clevers 2009) y las células troncales mesenquimales de la médula ósea (Gnecchi and Melo 2009). De acuerdo con nuestros resultados, otros estudios han descrito la expresión de genes específicos de linaje germinal como *Pou5f1*, *Dppa3*, *Ifitm3*, *Dazl* y *Ddx4* en la médula ósea en humanos y en ratones (Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Katsara, Mahaira et al. 2011). Además, *DAZL* ha sido localizado en células del fluido amniótico en humanos (Stefanidis, Loutradis et al. 2008, Stefanidis, Pergialiotis et al. 2013). Se ha propuesto que una población de células troncales embrionarias muy pequeñas ("very small embryonic stem cells", VSELs) reside en la médula ósea y en otros órganos adultos en el ratón y expresa varios marcadores específicos del linaje germinal. Estas células jugarían un papel funcamental en el reemplazo de las células multipotentes específicas de cada tejido y se originarían a partir del linaje germinal, siendo depositadas durante la gastrulación en los tejidos y órganos en desarrollo (Ratajczak, Shin et al. 2010).

En conclusión, además de la ampliamente conocida función de *Dazl* en el desarrollo de las células germinales, la expresión de *Dazl*-eGFP y de otros genes específicos del linaje germinal en otros tejidos que albergan células pluripotentes podría sugerir que las células troncales adultas comparten un origen común similar a una célula germinal con otras poblaciones de células pluripotentes cultivadas *in vitro* como las ESCs.

Presencia de células multipotentes en tejidos adultos y en líneas de células trofoblásticas bovinas

Algunos tejidos con una alta tasa de renovación celular como el intestino, la piel, el músculo esquelético o la médula ósea, son mantenidos por células multipotentes capaces de auto-renovarse durante largos periodos de tiempo, y de diferenciarse hacia un linaje celular específico (Macaluso and Myburgh 2012). Algunas de estas células han sido identificadas gracias a la expresión de marcadores de pluripotencia ampliamente conocidos como *Oct4*, *Sox2*, *Nanog*, *Klf4*, *Lin28* o *SSEA1*. Sin embargo, existen otros marcadores que son específicos de cada población de células multipotentes; por ejemplo, *Lgr5* de células troncales intestinales (van der Flier and Clevers 2009); CD48, CD150 o CD244 de células troncales hematopoyéticas (Kim, He et al. 2006); CD133, CD15 o CD24

de células troncales neuronales (Rietze, Valcanis et al. 2001, Capela and Temple 2002, Corti, Nizzardo et al. 2007, Peh, Lang et al. 2009); y CD10, CD13, CD73, CD105 o CD271 de células troncales mesenquimales (Mafi, Hindocha et al. 2011).

Además, algunos marcadores específicos del linaje germinal como *Dppa3*, *Ifitm3*, *Dazl* y *Ddx4* se han localizado en tejidos que albergan células multipotentes (Benson, Karsch-Mizrachi et al. 2004, Su, Wiltshire et al. 2004, Johnson, Bagley et al. 2005, Stefanidis, Loutradis et al. 2008, Katsara, Mahaira et al. 2011, Stefanidis, Pergialiotis et al. 2013). De la misma manera, en el capítulo III se encontró expresión de *Dazl*-GFP en médula ósea e intestino; y esto probablemente sea debido a las células troncales hematopoyéticas y mesenquimales que residen en la médula ósea, y a las células troncales intestinales localizadas en las criptas de Lieberhahn (Schabort, Myburgh et al. 2009). Por lo tanto, el transgen *Dazl*-eGFP podría usarse para analizar la presencia de células multipotentes en diferentes tejidos.

Las células troncales trofoblásticas (TSCs) conforman otra población de células multipotentes localizada en el trofoectodermo (TE) (Tanaka, Kunath et al. 1998). Sin embargo, las TSCs sólo se han aislado y caracterizado en profundidad en el ratón y en el macaco Rhesus (Vandevoort, Thirkill et al. 2007, Roberts and Fisher 2011). Por el contrario, en especies domésticas, debido a las diferencias en el desarrollo del TE (Bindon 1971, Geisert, Brookbank et al. 1982, Thatcher, Meyer et al. 1995), se desconoce la existencia y la localización de un nicho de TSCs. En consecuencia, las TSCs no han podido ser aisladas (Roberts and Fisher 2011), aunque se han obtenido líneas de células trofoblásticas en oveja y cabra (Miyazaki, Imai et al. 2002), cerdo (Ramsoondar, Christopherson et al. 1993, Flechon, Laurie et al. 1995, La Bonnardiere, Flechon et al. 2002) y bovino (Talbot, Caperna et al. 2000, Shimada, Nakano et al. 2001).

Curiosamente, las líneas de células trofoblásticas bovinas obtenidas en el capítulo IV muestran una capacidad de proliferación *in vitro* estable a largo plazo y expresan varios genes relacionados con la pluripotencia como *POU5F1, CDH1, FGF4, TERT*, y *TERC*. De acuerdo con nuestros resultados, otros estudios han sugerido que las células trofoblásticas no son células terminales y que presentan algunas propiedades de pluripotencia (Suzuki, Koshi et al. 2011, Schiffmacher and Keefer 2013). Por lo tanto, en las líneas de células trofoblásticas bovinas podría existir una población de células multipotentes responsable de estas propiedades.

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Conclusions

1. Embryonic quality determines ESCs derivation efficiency and transcriptional activity during early passages in mice, although differences disappear during long-term culture in optimal conditions. Thus, ESCs in early passages can be used as a model to analyze embryonic quality.

2. ESCs derivation in mice is conditioned by culture conditions and by proper expression of epigenetic markers, and the combination of LIF supplementation during embryo culture and posterior GS medium constitutes an efficient method for ESCs derivation.

3. Murine ESCs can emerge by two ways depending on culture conditions; through ICM-like or through epiblast or germline-like cellular subpopulations.

4. Germline-specific marker *Dazl* constitutes a pluripotency marker during development and in adult cells *in vivo* and *in vitro*, and can be used as a model to identify pluripotent cells in adult tissues.

5. Culture conditions determine bovine trophoblastic cell lines derivation efficiency from embryo biopsies, which allows preimplantation genetic and epigenetic diagnosis and the development of *in vitro* peri-implantational models.

Conclusiones

1. La calidad embrionaria determina la eficiencia de obtención de ESCs en ratón y su actividad transcripcional en pases celulares tempranos, pero estas diferencias no se mantienen estables en las ESCs, ya que el posterior cultivo en condiciones óptimas hace que desaparezcan. Por lo tanto, las líneas de ESCs en pases tempranos pueden ser usadas como un modelo para el análisis de la calidad embrionaria.

2. La obtención de ESCs en ratón está condicionada por los medios de cultivo utilizados y por la correcta expresión de marcadores epigenéticos, siendo la combinación de suplementación con LIF durante el cultivo embrionario y de medio de cultivo GS un método eficiente para su obtención.

3. Las ESCs de ratón pueden emerger utilizando dos vías; a través subpoblaciones celulares similares a las células de la ICM o bien a las del epiblasto o el linaje germinal, que son dictadas por las condiciones de cultivo.

4. El marcador de desarrollo germinal *Dazl* es también un marcador de pluripotencia tanto *in vivo* como *in vitro* y de linajes celulares embrionarios, fetales y adultos y ofrece un nuevo modelo para la identificación de células pluripotentes en tejidos adultos.

5. Las condiciones de cultivo determinan la eficacia de generación de líneas celulares de trofectodermo bovino a partir de biopsias embrionarias, abriendo las posibilidades del diagnóstico genético y epigenético preimplantacional, así como el desarrollo de modelos *in vitro* peri-implantacionales.

Curriculum vitae

Priscila Ramos Ibeas

Current research and skills

PhD student at INIA (National Institute of Agronomy and Food Research) in Madrid, Spain, defending my doctorate by July 2014. My current research concerns pluripotent cells isolation and *in vitro* culture, epigenetic, genetic and protein expression analyses. I am experienced in animal handling, embryo culture, cell culture, reprogramming and differentiation, bacterial culture, molecular biology (RNA and DNA extraction, RTPCR, qPCR, molecular cloning), epigenetics (bisulphite sequencing) and proteomics (immunohistochemistry, immunofluorescence, Western Blotting).

Education

2010-2014 (Defending date: july 2014)	PhD, Doctor in Veterinary Medicine Thesis title: "*Effect of origin and culture conditions on the heterogeneity of pluripotent cell populations*"	Complutense University, Madrid (Spain)
2009-2010	Master degree in "Research in Veterinary Sciences"	Complutense University, Madrid (Spain)
2004-2009	Degree in Veterinary	Complutense University, Madrid (Spain)

Work experience in research

Aug 2010 – Aug 2014	FPI grant from Spanish Ministry for PhD students	INIA (National Institute of Agricultural and Food Research and Technology), Madrid (Spain)
Oct 2008 - Jun 2009	Collaboration grant from Complutense University, Madrid (Spain)	Animal Health Department, Veterinary Faculty. Complutense University, Madrid (Spain)
Oct 2004 – Jun 2005	Academic excellence grant from Madrid Community (Spain)	Animal Production Department, Veterinary Faculty. Complutense University, Madrid (Spain)

Research stays

Sept 2013 – Dec 2013	Dr. Ramiro Alberio laboratory. Research in porcine epiblast stem cells and primordial germ cells.	The University of Nottingham (United Kingdom)
Jul 2012 – Nov 2012	Dr. Bhanu Telugu laboratory. Research in mouse, human and bovine induced pluripotent stem cells.	University of Maryland - USDA (United States)

Teaching experience

2011 - 2012	Master in Research in Veterinary Sciences	Complutense University, Madrid (Spain)
2010 - 2014	Reproduction seminars for Veterinary degree students	Complutense University, Madrid (Spain)
2010, 2011	XXXIII and XXXIV International Course of Animal Reproduction	INIA (National Institute of Agricultural and Food Research and Technology), Madrid (Spain)

Publications in indexed journals and book chapters

- "*An efficient system to establish biopsy-derived trophoblastic cell lines from bovine embryos*". **Ramos-Ibeas P**, Calle A, Pericuesta E, Laguna-Barraza R, Moros-Mora R, Lopera-Vásquez R, Maillo V, Yáñez-Mó M, Gutiérrez-Adán A, Rizos D, Ramírez MA. Biology of Reproduction. 2014. Paper in press BIOLREPROD/2014/118430.
- "*Intracytoplasmic sperm injection using DNA-fragmented sperm in mice negatively affects embryo-derived embryonic stem cells, reduces the fertility of male offspring and induces heritable changes in epialleles*". **Ramos-Ibeas P**, Calle A, Fernández-González R, Laguna-Barraza R, Pericuesta E, Calero A, Ramírez MA and Gutiérrez-Adán A. Plos ONE. 2014 Apr 17; 9(4):e95625.
- *"El coste de la inmortalidad: alteraciones genéticas y epigenéticas de las ESCs e iPSCs"*. **Ramos-Ibeas P**, Pericuesta E, López-Cardona AP, Laguna-Barraza R, Fonseca-Balvís N, Fernández González R, Gutiérrez-Adán A. Animales de Laboratorio. 2014. Accepted.

- *"Células troncales en vertebrados y sus aplicaciones al estudio y conservación de especies amenazadas o en peligro de extinción"*. **Ramos-Ibeas P**, Calle A, Ramírez MA. Animales de Laboratorio. 2014. Accepted.
- "*Most regions of mouse epididymis are able to phagocytose immature germ cells*". **Ramos-Ibeas P**, Pericuesta E, Fernández-González R, Ramírez MA, Gutierrez-Adan A. Reproduction. 2013 Oct 3; 146(5):481-9.
- "*The role of prion protein in stem cell regulation*". Miranda A, **Ramos-Ibeas P**, Pericuesta E, Ramirez MA, Gutierrez-Adan A. Reproduction. 2013 Jul 29; 146(3):R91-9.
- "*Sex-specific embryonic origin of postnatal phenotypic variability*". Laguna-Barraza R, Bermejo-Álvarez P, **Ramos-Ibeas P**, de Frutos C, López-Cardona AP, Calle A, Fernandez-Gonzalez R, Pericuesta E, Ramírez MA, Gutierrez-Adan A. Reprod Fertil Dev. 2012; 25(1):38-47.
- "*Solving the "X" in embryos and stem cells*". Bermejo-Alvarez P, **Ramos-Ibeas P**, Gutierrez-Adan A. Stem Cells Dev. 2012 May 20; 21(8):1215-24.
- "*Long-term and transgenerational effects of in vitro culture on mouse embryos*". Calle A, Fernandez-Gonzalez R, **Ramos-Ibeas P**, Laguna-Barraza R, Perez-Cerezales S, Bermejo-Alvarez P, Ramirez MA, Gutierrez-Adan A. Theriogenology. 2012 Mar 1; 77(4):785-93.
- "*Maintenance of Pluripotency in Mouse Stem Cells: Use of Hyaluronan in the Long-Term Culture*". **Ramos-Ibeas P**, Pericuesta E, Miranda A, Fernández-González R, Gutierrez-Adan A, Ramirez MA. Stem Cells and Cancer Stem Cells, Volume 7: Therapeutic Applications in Desease and Injury, pp. 123-134. Springer Science + Business Media Dordrecht, USA, 2012.

Submitted articles under revision

- "*Characterization of Dazl-GFP mice model generated by a two-step embryonic stem cells-based strategy to identify pluripotent and germ cells*". **Ramos-Ibeas P**, Pericuesta E Fernández-González R, Gutiérrez-Adán A, Ramírez MA. Submitted to Reproductive Biology and Endocrinology.
- "*Germ cell culture conditions facilitate the reprogramming to produce ESC in mouse*". **Ramos-Ibeas P**, Pericuesta E Fernández-González R, Gutiérrez-Adán A, Ramírez MA Submitted to Molecular Reproduction and Development.

Presentations in international congresses as first author

- "*A system to establish biopsy-derived trophoblastic cell lines for bovine embryo epigenotyping*". **Ramos-Ibeas P**, Calle A, Pericuesta E, Laguna-Barraza R, Moros-Mora R, Lopera-Vásquez R, Maillo V, Yáñez-Mó M, Rizos D, Ramírez MÁ, Gutiérrez-Adán A. COST EPICONCEPT Workshop. Epigenomic Toolbox: from Methods to Models. Gran Canaria, Spain. 2014.
- "*Paternal imprinting marks in mouse Spermatogonial Stem Cells are reset by overexpression of Yamanaka factors*". **Ramos-Ibeas P**, Bermejo-Álvarez P, Park K, Powell AP, Vansandt L, Ramirez MA, Gutiérrez-Adán A and Telugu BP. EPICONCEPT General Conference "Epigenetics and Periconception Environment" (COST). Antalya, Turkey, 2013.
- "*All the regions of the mouse epididymis are able to phagocyte immature spermatogenic cells*". **Ramos-Ibeas P**, Pericuesta E, Fernández-González R, Ramírez MA and Gutierrez-Adan A. 39th Annual Conference of the IETS. Hannover, Germany, 2013. Published in Reproduction Fertility and Development 12/2012; 25(1):272-3
- "*A Biopsy-Derived Trophectoderm Cell Line for Bovine Embryo Genotyping*". **Ramos-Ibeas P**, Moros-Mora R, Lopera-Vasquez R, Laguna-Barraza R, Gutiérrez-Adán A, Rizos D and Ramírez MA. 45th SSR Annual Meeting and 18th Ovarian Workshop State College, Pennsylvania, United States, 2012.
- "Dazl marks both pluripotent and germ cells". **Ramos-Ibeas P**, Pericuesta E, Fernández-González R, Gutiérrez-Adán A and Ramírez MA Cell Symposia: Stem Cell Programming and Reprogramming. Lisboa, Portugal. 2011.
- "*Arithmetic progressions in space; generalization of the traditional concept*". **Ramos Ibeas P**, De la Fuente C. Stockholm International Youth Science Seminar (SIYSS). Stockholm, Sweden. *2004*

Other contributions in international congresses

- "*Overexpression of Yamanaka Factors erases the imprinting marks of mouse Spermatogonial Stem Cells*". Bermejo-Álvarez P, **Ramos-Ibeas P**, Park K, Powell AP, Vandandt L, Ramirez MA, Gutiérrez-Adán A, Telugu BP. Stem Cells and Reprogramming, Keystone Symposia on Molecular and Cellular Biology. Resort at Squaw Creek, Olympic Valley, CA, United States. 2014
- "*Effect of embryo transfer on postnatal development and behaviour of mouse embryos*". López-Cardona AP, Fernández-González R, **Ramos-Ibeas P**, Alén F, Fonseca FR, Orio L and Gutiérrez-Adán A. EPICONCEPT General Conference Epigenetics and Periconception Environment (COST). Antalya, Turkey, 2013.

- "*Zrsr1 splicing factor controls spermatogenesis*". López-Cardona AP, **Ramos-Ibeas P**, de Frutos C, Pericuesta E, Calle A, Pintado B, Fernández-González R, Ramírez de Paz MA, Chitwood JL, Ross PJ, Gutiérrez-Adán A. Epigenetics for Improved Food Production: from Model to Practice. Girona, Spain. 2013
- "*The effect of embryo co-culture with different types of bovine oviductal epithelial cells and conditioned media in vitro on embryo development and quality*". Lopera R, Beltran P, **Ramos-Ibeas P**, Gutierrez-Adan A, Ramirez MA, Rizos D. 28th Annual Meeting A.E.T.E. Saint Malo, France. 2012
- "*Oxidative process and sperm epigenome*". Calle A, López-Cardona AP, **Ramos-Ibeas P**, Pericuesta E, Gutiérrez-Adán A. 2nd ISANH World Congress on Fertility & Antioxidants. París, France. 2012
- "*Parental age determines telomere length of the offspring in mice*". de Frutos C, Barraza-Laguna R, **Ramos-Ibeas P**, Rizos D and Gutierrez-Adan A. 4th COST Action FA0702 GEMINI General Conference Maternal Interactions with Gametes and Embryos. Gijón, Spain. 2011
- "*Germ cell culture-conditions facilitate the reprogramming to produce ESC*". Pericuesta E, **Ramos-Ibeas P**, Fernández-González R, Gutiérrez-Adán A and Ramírez MA. Cell Symposia: Stem Cell Programming and Reprogramming. Lisbon, Portugal. 2011

Communications in national scientific meetings

- "*Efecto de la restricción calórica aguda sobre el desarrollo folicular en conejas*". **Ramos Ibeas P**, García García RM, Lorenzo González P. V Jornadas complutenses. IX Congreso de Ciencias Veterinarias y Biomédicas. Madrid, Spain. 2010
- "*Efecto in vitro del interferón de tipo I en la expresión del virus de la inmunodeficiencia felina (FIV)*". Collado VM, Doménech A, **Ramos P**, Ballesteros N, Gómez-Lucía E. X Congreso Nacional de Virología. Salamanca, Spain. 2009
- "*Crecimiento y color de la carne de corderos ligeros suplementados con vitamina E*". **Ramos Ibeas P.** VI Congreso de Ciencias Veterinarias y Biomédicas. Madrid, Spain. 2007
- "*Paramyxovirus en mamíferos acuáticos*". **Ramos Ibeas P**, Rojas Solís P. V Congreso de Ciencias Veterinarias y Biomédicas. Madrid, Spain. 2006
- "*Estudio de la anestesia para la cesárea en perras*". **Ramos Ibeas P**. IV Congreso de Ciencias Veterinarias y Biomédicas. Madrid, Spain. 2005

Professional courses completed

- Cambridge English: Advanced (CAE). University of Cambridge ESOL Examinations. 2004
- Analytic and High Resolution Microscopy in Biomedicine. Basque Country University, Spain. February 2014.
- International Course in Animal Reproduction. INIA (National Institute of Agricultural and Food Research and Technology), Madrid (Spain). 2011

Academic prizes

First prize to early research works from Burgos University in 2004, to the research work entitled “Spatial arithmetic prograssions; a generalization of the traditional concept”.