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Review Article

Chicken embryonic stem cells as a non-mammalian embryonic stem cell model

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Embryonic stem cells (ESCs) were isolated in the early 1980s from mouse and in the late 1990s from primate and human. These cells present the unique property of self-renewal and the ability to generate differentiated progeny in all embryonic lineages both *in vitro* and *in vivo*. The mESCs (mouse embryonic stem cells) can contribute to both somatic and germinal lineages once re-injected into a recipient embryo at the blastocyst stage. In avian species, chicken embryonic stem cells (cESCs) have been isolated from the *in vitro* culture of early chicken blastodermal cells (cBCs) taken from stage X embryo (EG&K) These cESCs can be maintained under specific culture conditions and have been characterized on the basis of their morphology, biochemical features, *in vitro* differentiation potentialities and *in vivo* morphogenetic properties. The relationship between these cESCs and some of the chicken germ cells identified and grown under specific culture conditions are still under debate, in particular with the identification of the Cvh gene as a key factor for germ cell determination. Moreover, by cloning the avian homologue of the Oct4 mammalian gene, we have demonstrated that this gene, as well as the chicken Nanog gene, was involved in the characterization and maintenance of the chicken pluripotency. These first steps toward the understanding of pluripotency control in a non-mammalian species opens the way for the development and characterization of putative new cell types such as chicken EpiSC and raises the question of the existence of reprogramming in avian species. These different points are discussed.

Key words: blastoderm, chicken, germ cells, Nanog, Oct4, stem cells.

Totipotency and pluripotency in animal cells

Pioneer works carried out both in invertebrates (sea urchin) and vertebrates (newt) demonstrated as early as the last decade of the 19th century the existence of totipotent and pluripotent cells in early embryos. Indeed, Driesch (1891) demonstrated that a small sea urchin blastoderm dice will develop and form two independent animals whose size will be smaller than the parental one. This phenomenon is known as blastomere totipotency. Spemann replicated these results in 1902 with newt blastomeres taken at a two-cell stage and demonstrated that a newt larva could be split in two equal parts at 4, 8 or 16 blastomeres and regenerate two complete embryos. At this stage, the blastomeres are considered as pluripotent, that is, they are able to participate in the development of different embryonic tissues but not to reconstitute autonomously a whole organism.

The development of mammalian embryos takes place exclusively in the mother's body. After fertilization, the egg will enter into division and form a small cluster of cells called the morula. It will be compacted and the most outer cells will form the trophectoderm annexes, which will give rise to extra-embryonic tissues while the inner cell mass (ICM), derived from the inner part of the morula will form the future embryo. During this expansion process a cavity will be formed, the blastocyst, from the secretion of a liquid by the trophectoderm. The ICM will then form two derivatives, the epiblast (Epi) that will form the embryo and the primitive endoderm (PrE) that will give rise to extra-embryonic tissues. The implantation will take place and then the embryo will continue its development. Depending on the species, the kinetics of emergence of these early lineages are not the same.

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Mouse embryonic stem cells as embryonic stem cell archetype

The first mouse embryonic stem cells (mESCs) were isolated in 1981 from *in vitro* proliferation of the ICM derived from an E3.5 to E4.5 days post coitum (dpc) blastocyst (Evans & Kaufman 1981; Martin 1981). These mESCs are maintained undifferentiated under specific culture conditions on a feeder of irradiated mouse embryonic fibroblasts (MEFs). mESCs are small cells (8–12 μM) with a high nuclear cytoplasmic ratio that grow in aggregates. These cells have been progressively and extensively characterized. They exhibit an alkaline phosphatase (AP) activity, an endogenous telomerase activity and the same surface markers (SSEA1, ECMA7, EMA1, etc.) as the mEC cells do and present properties for *in vitro* differentiation. Progressively, the growing conditions have been improved including the use of feeder cells supplemented by EC conditioned medium (Smith & Hooper 1987) before the identification of the leukemia inhibitory factor (LIF) cytokine as the cytokine of choice to grow mESCs. Different cytokines from the gp130 family were then demonstrated to support self renewal of the mESCs including ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6), etc. (Yoshida *et al.* 1994). However, in mice, inactivation of the LIF cytokine is not lethal for early and late embryonic development, suggesting that the gp130 is not the only signaling pathway and that other molecules can maintain ICM cells (Ware *et al.* 1995; Yoshida *et al.* 1996; Kunath *et al.* 2007).

At the molecular level, these mESCs express a number of important transcription factors, such as *Oct4*, *Nanog* and *Sox2* whose respective functions start to be identified in details as described by several reviews (Masui *et al.* 2007; Niwa 2007; Niwa *et al.* 2009).

Using the mouse model, a general definition of embryonic stem cells (ESCs) may be formulated: these cells are capable, even after a long period of culture, to:

- self-renew indefinitely *in vitro*, without major alteration of their phenotype or their karyotype;
- differentiate *in vitro* into many cell types representative of the three embryonic layers: ectoderm, mesoderm and endoderm;
- participate *in vivo* in the formation of every cell in the embryo, including germ-line when they are reinjected into an early embryo;
- form tumors (teratocarcinomas) when injected into an adult organism.

At least in mice, it is possible to establish different pluripotent cell types from early embryos as well as from germ cells (EG cells), which retain similarities with embryonic cells (Matsui *et al.* 1992; Chambers & Smith 2004). More recently, pluripotent stem cells have been also isolated from E6.5 dpc mouse epiblast. Maintained in the presence of feeder with ActivinA and fibroblast growth factor 2 (FGF2) but in the absence of LIF, the so called EpiSCs were obtained and proliferate actively (Brons *et al.* 2007; Tesar *et al.* 2007). At the morphological level, these so called ''EpiSCs'' are more similar to the hESCs than to the mESCs. Despite a great potential of differentiation both *in vitro* with embryoid bodies (EB) and *in vivo* with teratoma formation, these EpiSCs are unable to form chimeras either through direct injection into blastocysts or using the morula aggregation protocol. Moreover, these cells are expressing genes such as *Dax1*, *Stella*, *PiwiL2*, *Stra8* and *Dazl*, which are also expressed in hESCs compared with mESCs (Tesar *et al.* 2007).

Beside these different ESCs, stem cells were also derived from the trophectoderm and primitive endoderm, as an example of extra-embryonic lineages. Trophectodermal stem cells (TS) can be derived from trophectoderm of E3.5 dpc embryos in the presence of feeder, heparin and FGF4 (Tanaka *et al.* 1998; Uy *et al.* 2002). The TS cells express specific transcription factors different from those of mESCs such as *Cdx2*, *Eomes* and *Hand1* but these cells do not express *Oct4*. Cells with close characteristics of the extra-embryonic endoderm (ExE) can be isolated from blastocysts at E3.5 in the growing conditions of the TS cells although FGF signaling is not required to maintain them in culture (Kunath *et al.* 2005). These cells, called XEN cells, present a peculiar morphology and express transcription factors also found in the extra-embryonic endoderm such as *Gata4*, *Gata6*, *Sox7*, *Hnf4* and *FoxA2* but again, do not express *Oct4*.

Figure 1 shows the different types of pluripotent cells that can be derived from the early mouse embryo.

Regarding this large range of stem cells obtained in a mouse model, an important step for both fundamental and applied research is to identify what would be the equivalent cell types in chicken, if they exist and if culture conditions would allow us to derive and amplify them for various purposes.

Pluripotency and isolation of embryonic stem cells in birds

In chicken, the egg is laid 20–23 h after fertilization. The early embryonic development that occurs in the oviduct was arbitrarily divided into 14 stages, numbered in Roman numerals (stages I to XIV), according to Eyal-Giladi & Kochav 1976 (Eyal-Giladi & Kochav 1976). The fertilized cell is undergoing rapid division to be laid at the so-called stage X embryo, which is

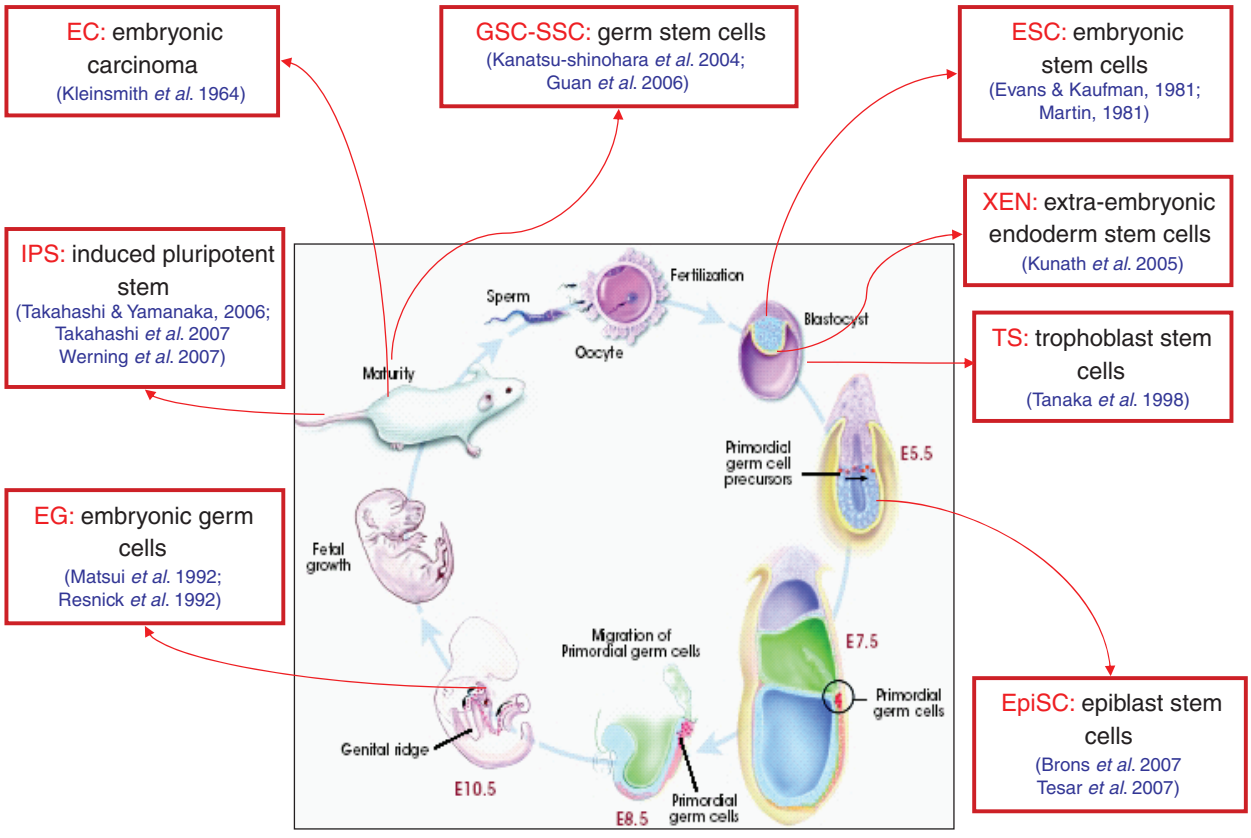

Fig. 1. Pluripotent embryonic stem cells (ESCs) were identified in mammals and in mouse in particular from various embryonic and adult tissues leading to the successive *in vitro* isolation of EC cells (Kleinsmith & Pierce 1964), of ESCs (Evans & Kaufman 1981; Martin 1981), of trophectodermal stem (TS) cells from trophectoderm tissue (Tanaka *et al.* 1998), of XEN from extra embryonic endoderm cells (Kunath *et al.* 2005), of EpiSCs from epiblast (Brons *et al.* 2007; Tesar *et al.* 2007). Germinal derivatives provided by EG cells from germinal embryonic ridges (Matsui *et al.* 1992; Resnick *et al.* 1992) and germ stem cells (GSC) from differentiated spermatogonial cells (Kanatsu-Shinohara *et al.* 2005; Guan *et al.* 2006). Finally, reprogrammation led to the obtention of the so-called ''iPS'' (induced pluripotent stem cells) from somatic cells genetically modified with defined transcription factors (Takahashi & Yamanaka 2006; Takahashi *et al.* 2007; Wernig *et al.* 2007) . Scheme adapted from National Institute of Health (NIH) stem cells report, – 2000.

already composed of 20 000–50 000 cells, called blastodermal cells (BC). Morphologically, this stage X embryo can be divided into area opaca at the peripheral part and the area pellucida representing the central part of the embryo. The entire embryo and some of the extra-embryonic tissues derive from the epiblast, which is separated from the yolk by the subgerminal cavity. Secondary hypoblast will be set up by delamination from the epiblast and hypoblast cells are in direct contact with the yolk and divisions are not completed. Once incubated, the epiblast cells will undergo a number of morphogenetic movements enabling the establishment of the primitive streak, the future axis of the embryo. The embryonic development after laying was arbitrarily divided into 46 stages printed in Arabic numerals (stages 1–46) according to Hamburger and Hamilton Table, established in 1951 (Hamburger & Hamilton 1951).

An important step was achieved by Spratt and Haas (1961). These authors demonstrated the pluripotent nature of the blastodermal cells by dividing a blastoderm in four equal parts, each of them was therefore able to give rise to a normal embryo. Furthermore, it was demonstrated that blastodermal cells could be injected and colonize a recipient embryo (Marzullo 1970). From cell clusters derived from freshly laid non-incubated embryos, chimeras (3 on 239) were obtained, based on colored feathers. However, no live chick hatched. No new results were recorded until 1990 when Pettite and colleagues slightly modified the process of obtaining chimeras, mainly by dissociating the blastodermal cells. They were able to obtain live chimeras from stage X blastodermal cells and in particular to get a germ line chimera, that is, one that is able to transmit to his progeny the genotype of the injected cells, demonstrating for the first time the germ

line competency of the injected cells and therefore the germ line competency of the stage X blastodermal cells. Slight irradiation of the recipient embryos increased both the yield of somatic chimerism to 15–20% as well as the germ line chimerism around 3% (Carsience *et al.* 1993). This somatic and germ line colonization demonstrated that stage X embryonic cells were pluripotent.

On this basis, we decided to isolate *in vitro* growing cells harboring ESC features and to characterize them as illustrated (Fig. 2). We were the first to establish *in vitro* culture of chicken embryonic stem cells (cESCs) (Pain *et al.* 1996). The blastodermal cells (cBCs) were derived from a fertile non-incubated stage X embryo and maintained as their murine counterparts on a feeder of irradiated embryonic fibroblasts. It has to be noted that different fibroblasts were tested such as primary mouse fibroblasts (MEFs), established STO line (a mouse embryonic fibroblast cell line) as well as avian cells including primary chicken embryonic fibroblasts (CEFs), quail established QT6 line, etc. Among the different tested feeders, the STO provided the most steady and reproducible results once cBC were plated on it. A cocktail of cytokines initially composed of recombinant LIF, Interleukin 11 (IL-11), stem cell factor (SCF), insulin growth factor 1 (IGF1) and basic fibroblast growth factor (bFGF) ensured their initial proliferation in a non-differentiated status. More simple combinations have since been established, especially by the use of the IL6 and IL6Rαs as a key component of the gp130 signaling pathway. Moreover, we observed that the presence of LIF added during initial plating of the cBC enhances the ability of the cells to become established. Recent advances showed that the maintenance of cESC can be solely dependent on the presence of avian LIF and serum (Horiuchi *et al.* 2004). However, it was not established whether this cytokine is able to provide cESC culture competent for either somatic and/or germ line contribution as no chimera experiments were reported.

It has to be mentioned that the genetic background of cESC isolation is not well documented. Indeed, different strains were tested for their ability to provide cESCs in culture, and we observed differences in the initial growth and establishment efficiency including differences between egg-producing strains and broilers with apparently a greater ease of establishment in the broilers. As relationships between the different strains of chicken – from a genetic point of view – are not so well described and identified, it was rather difficult to assess the ability to obtain growing cESCs from one or more characters. Figure 3 illustrates the growth and establishment of the cESCs comparatively to CEF in two different genotypes.

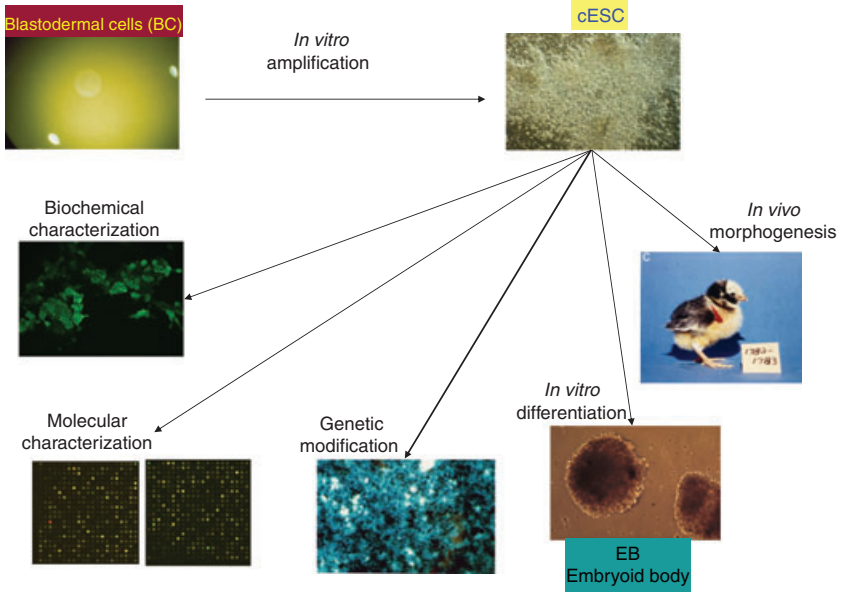

Fig. 2. Isolation of chicken embryonic stem cells (cESCs) was carried out from *in vitro* culture of blastodermal cells taken from stage X (EG&K) embryos. Plated under specific described conditions of feeder, serum, growth factors and cytokines, cESCs can be amplified and were successively characterized at the biochemical level, by their *in vitro* differentiation potentialities and by their ability to contribute to morphogenesis once injected back into a recipient embryo. Germinal contribution was present but rarely observed for long term culture cells. The cESCs have been successfully genetically modified by various kinds of factors and are presently submitted to a molecular characterization using the available chicken-specific pangenomic microarrays.

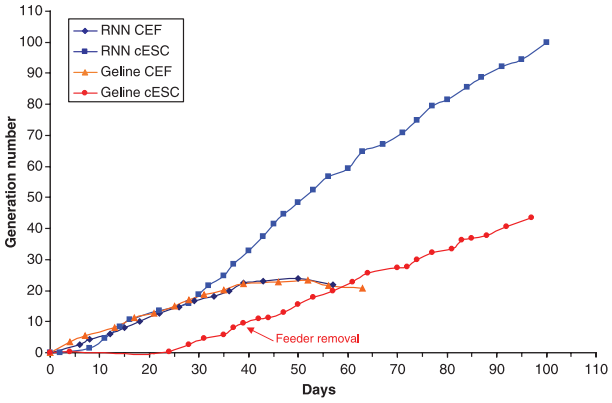

Fig. 3. Once plated in *in vitro* culture, the stage X blastodermal cells start to proliferate and become established after various period of times depending on their genetic background. In contrast to the chicken embryonic fibroblasts (CEFs), which are entering into senescence after 10–15 passages (roughly corresponding to 20 cell generation), chicken embryonic stem cells (cESCs) continue to proliferate actively in the absence of any exogenous transforming agent and after a few passages become established and maintain a more or less constant proliferation rate. As illustrated for the two distinct genetic backgrounds, the RNN (Red Naked neck strain) and the local geline strain, different growth rate could be obtained depending on the cell isolate. On this particular curve, the presence of the initial feeder cells was removed after a few passages for the geline cESCs leading to the obtention of a feeder-free cESC established culture. These cells present similar features compared with the routinely feeder-maintained cESCs. (–♦–) RNN CEF, (–■–) RNN cESC, (–▲–) Geline CEF, and (–●–) Geline cESC.

Biochemical characterization

Different markers are routinely used to characterize cells and control the culture conditions of long-term maintained cESCs (Table 1). In particular, classical antibodies including SSEA-1, SSEA3 and SSEA4 are used. These antibodies were raised against mouse EC cells and were demonstrated to recognize different ES cells from various species. We were the first to describe a cross-reactivity of these antibodies with chicken cells derived from cultured blastodermal cells. In particular, reactivity toward both SSEA1 and SSEA3 is a chicken specificity as mESCs are positive for SSEA1 labeling but not for SSEA4 and hESCs are, in contrast, negative for SSEA1 and positive for SSEA4. The presence of intense alkaline phosphatase activity is also a common and rapid way to look for the good culture conditions. Like their mammalian counterparts, these cESC can be grown *in vitro* for long-term culture without exhibiting senescence or growth crisis. The presence and maintenance of endogenous telomerase activity is likely responsible for this long term establishment (Pain *et al.* 1996). After this first demonstration of a telomerase activity in chicken cells, the chicken telomerase gene was cloned (Delany & Daniels 2004; Swanberg *et al.* 2004). In contrast, as soon as the cESCs are induced to differentiate, this endogenous expression is rapidly downregulated (Lavial *et al.* 2007).

Table 1. Main features of embryonic stem cells (ESCs) from different species

	mESC	hESC	cESC
AP	++	++	++
SSEA1	++	–	++
SSEA3	–	–	++
SSEA4	–	++	–
Tert	++	++	++
Oct4	++	++	++
Nanog	++	++	++

AP, alkaline phosphatase; cESC, chicken embryonic stem cells; hESC, human embryonic stem cells; mESC, mouse embryonic stem cells; SSEA1, SSEA3, SSEA4, stage specific embryonic antigen 1, 3 and 4; Tert, telomerase activity, *Oct4* and *Nanog* gene expression.

Genetic modification

We have previously shown that cESCs could be genetically modified by various vectors including simple expression vectors (Pain *et al.* 1999), gene trap vectors (Acloque *et al.* 2001) and homologous recombination vectors (Pain et al. unpubl. data, year, 2001; patent application FR N01/15111). For simple expression vectors, different promoters are active in the non-differentiated cESCs, as well as differentiated cESCs. In particular, strong promoter such as viral cytomegalovirus (CMV) promoter or chicken b-actin derived promoter such as the CAGG promoter (CMV immediate-early enhancer and the chicken β-actin/rabbit β-globin hybrid promoter (Niwa et al., 1991)) are often used to overexpress transgenes in the proliferating cESCs. Retrovirus infections have been demonstrated to be efficient *in vitro* on these cells (Pain et al., unpubl. data, 2006) as well as *in vivo* infection on blastodermal cells present in fertile embryos (McGrew *et al.* 2004).

In vitro differentiation potentialities

As for their murine counterparts, different methods were developed to demonstrate and control the *in vitro* differentiation potentialities of the cESCs.

One approach is to plate cESCs in a culture medium containing no cytokines and growth factors needed to sustain cell proliferation. In particular, the absence of one of the family cytokines gp130 (LIF, IL-6, CNTF, GPA, IL-11, etc.) leads to a slowdown in proliferation

and a progressive loss of the pluripotency markers. However, the differentiation of cells submitted to this process is not homogenous, and different cell types will be obtained depending on the initial plating density, the expected autocrine and paracrine cell production and the influence of the cells to each other. The heterogeneity of the cells obtained through this non-controlled process can be estimated by detecting the presence of early lineage markers, such as *Brachyury* and *Goosecoid*, specific to mesendoderm lineage, *Sox1*, *Sox2*, and *Pax6*, markers of neurectoderm lineage and *Gata4*, *Gata6*, *Hnf*, three markers specific to the endoderm lineage (Lavial *et al.* 2007).

A second approach involves the production of EBs by seeding the cells in a bacterial dish. The cells are trypsinized and cultured in non-adherent suspension either in a large volume under slow agitation or in a small volume (20–50 μL) in hanging drops. All of these cultures are carried out in a serum-depleted medium (from 0.5% to 5.0% serum for example) and in the absence of growth factors and cytokines required for normal non-differentiated proliferation. By preventing the cell adhesion and basolateral polarization, embryonic stem cells differentiated by adopting a three-dimensional structure that mimics the embryonic lineages. Once formed after 2–5 days, EBs can be plated again to obtain more fully differentiated cells in the presence of specific growth factors or used directly for further characterization.

A third approach is the use of chemical inducers to reinforce the cESCs differentiation. By chemical induction, it means the use of any non-peptide chemical molecules, whether natural or obtained by chemical synthesis. For example DMSO (dimethylsulfoxide) is often used as a general inducer generating a mixed differentiated cell population with several cell types. In contrast, retinoic acid usually allows a more rapid differentiation. As an illustration, these different inductions were used to demonstrate that the chicken *Oct4* and *Nanog* gene expression was downregulated following the induction of cESC differentiation (Lavial *et al.* 2007).

A forth approach is to silence a specific gene expression by the use of conditional shRNA. Taking advantage of the system used in mouse (Coumoul *et al.* 2004), we developed this innovative approach in chicken cells by using a conditional floxed U6 promoter to direct the expression of shRNA against different genes including pluripotent associated genes. By inducing their expression, we obtained cESCs induced to differentiate into a cell type that is still under investigation at the transcriptomic level but that did not proliferate as their parental cells. Moreover, the gene expression profile clearly demonstrates that such inactivation leads to the loss of pluripotent markers. Basically, a SHRNA and Neomycine cassette containing expression vector is stably transfected into cESCs. Resistant clones emerge, expand and are submitted to a second round of transfection with a CRE-ERT2 expression vector (Feil *et al.* 1997). Following a second selection (hygromycin for example), amplified clones are submitted to the tamoxifen action to induce the recombinase activity. Tamoxifen addition induces the excision of the floxed resistance cassette and allows the shRNA expression leading to a strong phenotypic change observed as soon as 48 and confirmed 96 h after addition of drug (Lavial *et al.* 2007). This approach is particularly important for genes involved in cell pluripotency control, cell cycle or terminal differentiation, etc.

Another approach is to use enrichment protocols with positive genetic selection after cESC transfection. For this, expression of a resistance gene (neomycin, hygromycin, puromycin, zeomycin, blasticidin, etc.) or of a phenotypic marker including fluorescent proteins such as green fluorescent protein (GFP), is placed under the control of a developmental stage or tissue-specific promoter. Once induced to differentiate, the cells expressing this resistance and/or this marker can be enriched by this particular way. Among these positive strategies, overexpression of a specific cDNA of interest is capable of inducing differentiation in a given lineage. In particular, genes such as *MyoD* for example or other related key master genes controlling lineages can trigger terminal differentiation of cESCs once transfected (Pain, unpubl. data, 1998).

In vivo chimerism

To test the potential contribution to the development of mESCs, microinjection of these cells into a blastocyst has been developed for many years (Gardner 1968). Dissociated cells are directly injected into the blastocoelic cavity of an E3.5 dpc embryo or aggregated with an embryo at the morula stage. However, this last technique cannot be extended to the blastocyst stage.

In chicken, the structure of the recipient blastoderm is slightly different. One advantage is that the cells can be directly injected into the embryo and that no foster mother is required. Different studies have demonstrated the ability of the blastodermal cells (cBCs) to participate in the morphogenesis and the germline of the embryos (Carsience *et al.* 1993). We have demonstrated that cultured cESCs can produce high level somatic chimeras and sometimes germline chimeras. However, we and others have also found that this germline competency becomes very low and almost

lost when cells are cultured for a long period. These results suggest that the germinal competent cells are either lost during the culture process as they cannot proliferate or the culture conditions are unable to maintain them, leading in both cases to a loss of germline competency (Pain *et al.* 1996; Petitte *et al.* 2004; van de Lavoir *et al.* 2006b). In all cases, injection into a recipient embryo is usually carried out in stage X embryo. that is, non-incubated embryo when cells are not completely induced. Another approach is to inject the cells directly into the blood stream of an incubated embryo as used and described by Simkiss (1997) and Naito *et al.* (1994). This method could provide a better homing for germ cells as these last cells migrate through the blood stream to reach the gonads during a small window of time.

Can EpiSCs be obtained from early chicken embryo?

With the isolation of mEpiSCs derived from late embryonic epiblast, the question of the existence and isolation of similar cells in chicken is still opened in particular, as gastrulation occurs very rapidly after laying once the embryo is incubated. Indeed, on the model of the different stem cells isolated in the mouse, it can be hypothesized that similar cells are present and can be isolated in chicken (Fig. 4). It appears indeed that the mEpiSCs are more similar to the hESCs rather than to the mESCs in terms of molecular signature. At the mRNA expression level, mEpiSCs express a number of genes associated with the germline such as *Stella*, *PiwiL2*, *Stra8* and *Dazl*, which are almost undetectable in mESCs (Tesar *et al.* 2007). Moreover, mEpiSCs express also trophectoderm markers such as *Eomes* and endoderm markers, such as *Gata6* and *Sox17* (Russ *et al.* 2000). In our hands, cESCs express pluripotency associated genes such as *cPouV*, *cNanog*, *cSox2* and *Fgf4* as well as some of the Krüppel-like factors such as *Klf2*, suggesting these cells could be true ESCs according to the recent comparison between mESC and mEpiSC (Nichols & Smith 2009). However, these proliferating cESCs also express the Activin receptor (*AcvR2B*), the chicken homologue of *Eomes* as well as some germinal-associated genes including *Piwi*, *Stra8* and *Tudor*. Taken together, these preliminary data suggest that the chicken molecular signature of the cESC is similar to the mESC in terms of pluripotency-associated markers but with noticeable exceptions including the presence of *Eomes* for example (Lavial *et al.* 2007; Lavial *et al.*, unpubl. data, 2008). However, developmental processes appear to be quite different between mouse and chicken and could make comparisons quite difficult between these different cell populations regarding the significance of some specific gene expression profiles. In particular, the initial heterogeneity of the blastodermal cells usually observed from stage IX–XI early embryos should be considered more carefully and avoided to obtain cESC cell cultures derived from one well-defined stage only. In this perspective, it would be highly informative and interesting to try to derive some *in vitro* cell culture of ''EpiSC-like'' in chicken from older than stage IX–XI (EG&K) embryos including stage XII–XIV pre-gastrulating embryos. Moreover, the addition of growth factors such as Activin and FGF either on stage IX–XI or on stage XII–XIV blastodermal cells could generate new chicken pluripotent cells with different properties and molecular signatures. Among them some EpiSC-like cells could be found. Presently, more data are required at both cellular and molecular levels to conclude on the identity and relationship between presently established cESCs and the putative chicken EpiSC-like cells, if they exist and if they can be isolated and propagated.

Can chicken iPS be obtained following somatic reprogrammation?

The chicken model is still a powerful model for early developmental investigations (Stern 2005) and the use of the cESCs is also seen as a very useful tool to compare and develop phylogenic approaches in the stem cell field (Pain *et al.* 1996; Petitte *et al.* 2004). The identification of mammalian iPS following somatic cell reprogramming (Takahashi & Yamanaka 2006; Takahashi *et al.* 2007; Wernig *et al.* 2007) raises the question of the existence of a similar process in chicken that could give rise to ''cIPS''. Presently no evidence supports this possibility even if the recently identified chicken genes *cPouV* and *cNanog* by our group were demonstrated to be key actors in the maintenance of chicken pluripotency. Such genes, as well as specific chicken genes, *cSox2*, *cMyc* and a chicken *Klf* gene, could serve as a molecular basis for such experiments. Interestingly, the *c-Myc* gene is a well-known factor in the avian field as the *v-Myc* oncogene was identified in the MC29, a natural avian retrovirus and the transformation potential of the different *c-Myc* and *v-Myc* forms were described in great detail (Farina *et al.* 1992; Li *et al.* 1996). In regard to papers demonstrating a somatic reprogrammation without the use of the active proto-oncogene *c-Myc* (Nakagawa *et al.* 2008), even if the process appears to be more difficult, it would be highly informative to compare the presence or the absence of the different Myc genes (*c-Myc*, *N-Myc* and *L-Myc*) from both avian and mammalian origin during the reprogramming process. Indeed, the

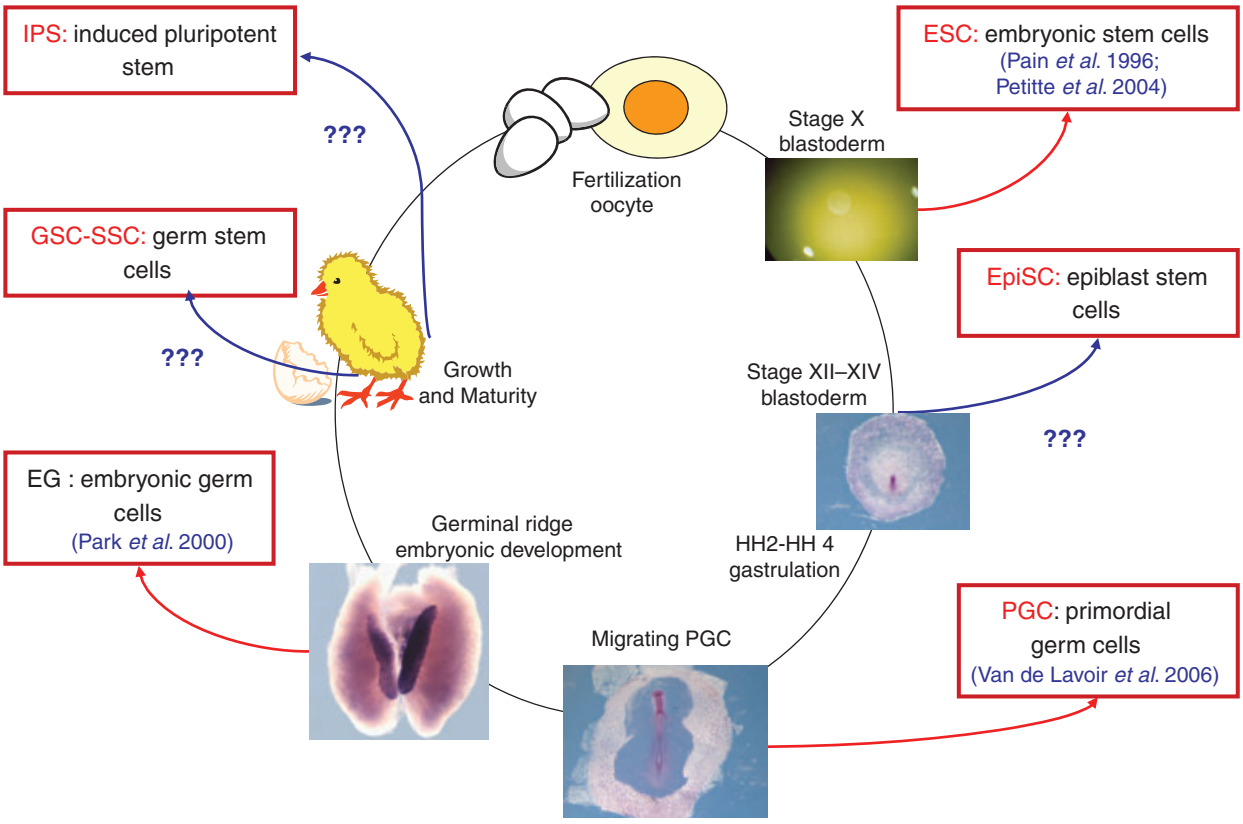

Fig. 4. In chicken different stem cells have been also identified, isolated and grown *in vitro*. From stage X blastodermal cells (BC), chicken embryonic stem cells (cESCs) were isolated and amplified *in vitro* using a combination of growth factors and cytokines (Pain *et al.* 1996; Petitte *et al.* 2004). EG cells from gonadal cells were isolated using endogenous gonadal feeder cells and growth factors and cytokines similar to those used for cESCs (Park *et al.* 2003). Taking advantage of the circulating primordial germ cells (PGCs) in early embryonic blood, PGCs have been established in culture using cytokines and culture conditions partially different from those used to amplify cESCs (van de Lavoir *et al.* 2006a). Questions remain opened regarding the identification, isolation and growth of putative EpiSCs from older embryos than stage X embryos, of germ stem cells–spermatogonial stem cells (SSCs–GSCs) from embryonic and/or adult gonadal germ cells and spermatogonia. Moreover, isolation and obtention of chicken iPS is also. Is presently hypothesized as somatic reprogramming by genetic modification with transcription factors was not yet demonstrated in other species than mammals.

identification of the right gene combination with different or new candidates could contribute to the identification of the reprogramming mechanisms. Moreover, culture conditions defined by the addition of either epigenetic chemical modifiers or specific signaling pathway inhibitors could also participate to define and control this process, which is still inefficient in mammals and not yet described in avian species. Therefore, some of the main scientific challenges to be addressed are the identification of the molecular factors controlling the reprogrammation process in avian, a non-mammalian species and the description and characterization of the epigenetic changes that could occur during this process in chicken. Phylogenetically, understanding how an avian somatic cell would acquire a stem cell phenotype by reprogrammation would be particularly important at both fundamental and applied levels.

Germ line competency and *in vivo* contribution of cESCs

Relationship between embryonic stem cells and germ line competency is still an open field of investigation. Then, it is still unknown under what culture conditions the cESCs would be able to keep long term germinal competency once maintained in long term *in vitro* culture. Indeed, germ cells retain the ability to form a new individual and must therefore not respond to differentiation signals from surrounding cells during development. Schematically, the establishment of the germ line is associated with the suppression of somatic differentiation in many organisms. Two main ways of germ line formation are identified: the preformation and the induction models (Extavour & Akam 2003). In chicken, it is hypothesized that the germ line determination is based on the preformation model. In particu-

lar, the presence of Cvh positive cells in stage X blastodermal cells and even earlier sustains this hypothesis (Tsunekawa *et al.* 2000).

In *Caenorhabditis elegans* and *Drosophila melanogaster* at the pluripotent zygote stage, precursors of the germ line called PGCs (primordial germ cells) are specified by maternal components present in the cytoplasm. Asymetrical division induces this cell determination and different genes including *Pie1* for *C. elegans* and *Gcl* for *D. melanogaster* are among the factors controlling the transcriptional repression leading to this complex germ cell (Strome & Lehmann 2007). In mice, the PGC appear under the inductive model. Indeed, some cells in the proximal epiblast will receive signals, mainly BMP4, BMP8b from the extra-embryonic ectoderm and the visceral endoderm. These primed cells will become competent to form germ cells, highlighted by the expression of the Fragilis early marker. A key event of the PGC determination is the expression of transcriptional repressor *Blimp1*, which exhibits a histone methyltransferase activity and which is associated with various chromatin modifiers such as *Prmt5* (Ohinata *et al.* 2005; Ancelin *et al.* 2006). Indeed, some of the cells expressing *Fragilis* become *Blimp1* positive and are rapidly engaged in germinal differentiation, while adjacent cells are engaged in somatic differentiation (Saitou *et al.* 2002; Hayashi *et al.* 2007). It seems that *Blimp1* represses the expression of *Hox* genes and therefore a program of somatic differentiation. Moreover, after their specification, the PGCs undergo a strong epigenetic reprogramming associated with the loss of H3K9 dimethylation, an increase of H3-K27 trimethylation as also observed in *C. elegans* and a loss of DNA methylation (Seki *et al.* 2007; Hajkova *et al.* 2008).

Even if there are differences between models, a number of mechanisms are kept at the establishment of the germ line, in particular a global repression of transcription and an overall chromatin remodeling (Surani *et al.* 2007).

In chicken, debate remains open as to whether the appearance of germ cells occurs following a predetermined process. Indeed, from the stage X embryo (EG&K), clusters of cells expressing surface antigens such as SSEA-1 and EMA-1 and also some specific germ cell markers such as *Cvh* (chicken vasa homologue) and *Dazl* are detected as early as this non-incubated stage. These markers, as well as the presence of strongly alkaline phosphatase positive cells, are also detected in the germinal crescent after 18 h of incubation (Urven *et al.* 1988; Karagenc *et al.* 1996; Tsunekawa *et al.* 2000; Naito *et al.* 2001).

Experimental ablations of the germinal crescent at 7–10 (H&H) show that this embryonic structure is the sole source of PGCs of the chicken embryo. One of the most distinctive features of chicken germ cells is their transient presence in embryonic blood as circulating PGCs during a few hours (from 48 to 55 h of development) between stages 14 and 17 (H&H) just before their arrival in the undifferentiated gonads. Only the cells that arrive to these gonadal structures will differentiate into germ cells. The other cells enter into apoptosis as they did not find favorable proliferation conditions. Steel factor/c-Kit as well as SDF-1/CXCR4 play an important role for such guidance even if fine molecular mechanisms still need to be identified including a specific role for genes such as *Dnd* (*Deadend*) (Doitsidou *et al.* 2002; Weidinger *et al.* 2003).

In mouse, different cells with pluripotency associated properties were isolated from germ cells. Initially identified by the strong expression of alkaline phosphatase, the cells are detected as early as E7 dpc at the junction between epiblast and extra-embryonic ectoderm (Lawson & Hage 1994). Until E12.5, they migrate and proliferate to form a population of around 25 000 cells. At that time, the PGC can be isolated from the genital ridge of E8.5 to E12.5 dpc embryos and a combination of SCF, LIF and bFGF allowed the growth of PGCs that become EG cells (embryonic germinal cells) (Godin *et al.* 1991; Matsui *et al.* 1991). The EG cells present very similar characteristics as the mESCs in terms of teratoma and chimera formation (Matsui *et al.* 1992; Resnick *et al.* 1992; Labosky *et al.* 1994). However, they present differences in terms of methylation of some loci such as *Igf2R*.

Using another combination of growth factors and cytokines, mainly GDNF, EGF, LIF and bFGF, germ stem cells (GSCs) were established from newborn mouse testes (Kanatsu-Shinohara *et al.* 2005; Kanatsu-Shinohara & Shinohara 2007). These cells were stem cells but different from mESC and mEG cells in terms of differentiation potentialities. Pluripotent germ stem cells were also derived from adult mouse testes (maGSC) (Guan *et al.* 2006) using a genetic enrichment procedure and spermatogonial stem cells (SSCs) were isolated using the presence of GFP under the control of the Stra8 promoter, known to be expressed in spermatogonia (Oulad-Abdelghani *et al.* 1996) (Fig. 1).

In chicken, different attempts have been made to isolate and grow germ derived cells. Isolated from the embryonic blood during their migration, PGC can be maintained under non-differentiated conditions in the presence of cytokines such as LIF, SCF and bFGF (van de Lavoir *et al.* 2006a). According to the authors, these cells are round, do not attach to the substrate and form germ line chimeras at a high frequency when

injected into stage 13–15 (H&H) embryos, but do not participate in the somatic tissues, even if they are injected into early stage X (EG&K) embryos. Moreover, when transferred into a medium with a decreased level of serum and the absence of FGF, these cells turned into adherent cells presenting EG characters. However, surprisingly, these EG-derived cells do not contribute any more to the germ line even if they are, at that point, able to contribute to somatic tissues (van de Lavoir *et al.* 2006a).

When taken directly in the developing gonads from a stage 28 (H&H) embryo, the germ cells can be established *in vitro* under culture conditions including the use of LIF, SCF, FGF2, IGF-1 and IL-11 highly similar to those conditions we developed for cESCs (Pain *et al.* 1996). This protocol led to the establishment of EG-like cells obtained from scattered embryonic gonadal cells taken from a 5.5 day old incubated embryo (Park & Han 2000; Park *et al.* 2003). The establishment is a two step process including a first phase of colonies formation of gPGCs (gonadal PGCs) directly on stroma cells derived from the plated gonads that progressively turn into a more homogenous culture when these proliferating PGCs are plated in a second phase on irradiated feeder cells. These cells have the ability to differentiate both *in vitro* by forming EBs and *in vivo* by contributing to somatic chimeras when injected into stage X (EG&K) embryos. Injection experiments in stage 17 (H&H) embryos demonstrated that these cells are able to colonize gonads of the recipient embryos and give rise to germ line chimeras. However, this ability is also rapidly lost with long term cultures, greatly limiting the use of such cells for biotechnological approaches (Park *et al.* 2003).

A key question remains open regarding the presence of the genes controlling the germinal competency of the different cultured stem cells and the particular relationship between PGCs and cESCs. However, the lack of germ line competency of the isolated cells could be due to the molecular differences between them with definitive loss of germinal potentialities but also to the inability of some cells to colonize the embryo or gonads when reintroduced into embryos as the different developmental stages between the donor cell and the recipient do not match. Presently, no large scale analysis has ever described and compared systematically the ability of either stage X BC, cESC, PGC or EG cells to colonize the different tissues and gonads when reintroduced either into a stage X embryo, or into blood stream or into just hatched chick gonads. This combinatory analysis, -which appears probably difficult to realize for practical reasons could, however, reveal some efficient and inefficient combinations between host and injected cells, leading to a better choice of the model to reveal the potentiality of a defined cell.

Regarding the molecular events, we have started to investigate further into the details of the molecular relationships and the differences that are responsible for the progressive loss of germinal competency of the cESCs. In contrast to the PGCs, the germ cells taken from gonads as well as the initial cBC cells, we have indeed found that *in vitro* cultured chicken cESCs are expressing reduced levels of the *Cvh* marker and no expression at all of the *Dazl* gene (Lavial *et al.* 2009). However, by overexpressing these genes into cESCs, we found that *Cvh* was able to restore a germline competency of the modified cESCs by both impairing the somatic differentiation program and inducing the germinal one. A fine balance between the level of expression of the pluripotency associated genes and the germline-specific genes is observed as overexpression of *Cvh* implies a downregulation of the *cPouV* and *cNanog*. This would also be important to define the requirement of these pluripotent associated genes for germ cell determination and survival in chicken as demonstrated in the mouse for Oct4 (Kehler *et al.* 2004) and Nanog (Chambers *et al.* 20, 2007; Yamaguchi *et al.* 2005). Further experiments should be carried out to define the gene network controlled reciprocally by both pluripotent and germline-specific factors. Presently and interestingly, the *Dazl* gene appears to be unable to mimic the *Cvh* effect, but the level of expression of the endogenous *Dazl* gene is therefore strongly upregulated following the expression of the exogenous *Cvh*. This unique combination seems to restore by itself the ability of the cells to colonize more efficiently the gonads and to contribute to the germinal lineage. We are now proposing that the expression level of these two main germinal markers were delimiting a germline boundary of the different chicken stem cells that have been presently reported as illustrated in Figure 5.

Conclusion

In conclusion, embryonic stem cells are mainly studied in mammals, initially in mouse and increasingly in human for the exciting perspectives opened by the future potentialities of regenerative therapies of these cells. Regarding the non-mammalian species, some stem cells have indeed been identified. In particular, we demonstrated that cESCs can be identified and amplified *in vitro* with specific ESC features. Despite the lack of complete molecular characterization, we already identified *cPouV* and *cNanog*, as two of the key factors controlling chicken pluripotency, and some of the elements controlling the germinal com-

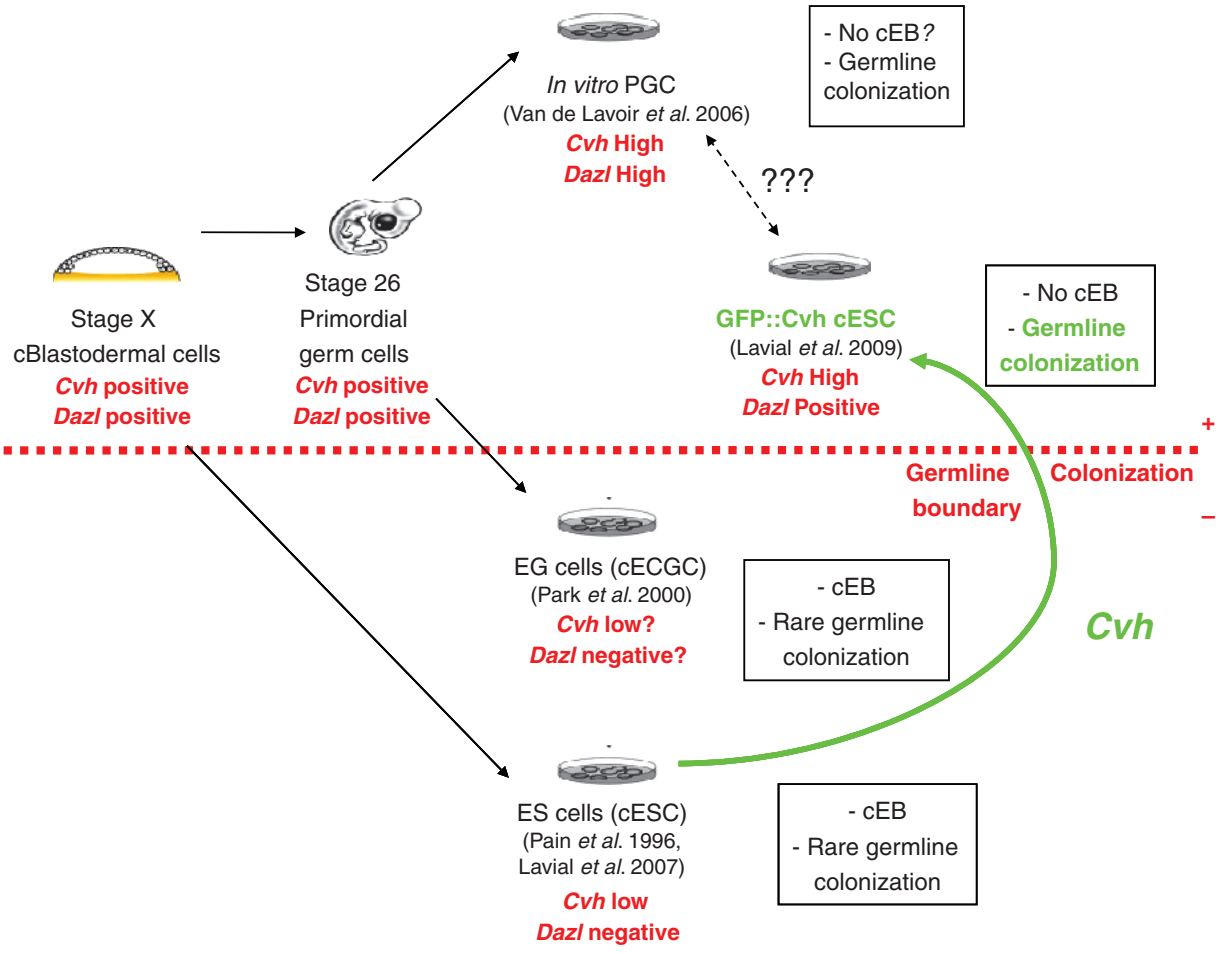

Fig. 5. Relationship between pluripotency and germ line competency is a central question for the chicken stem cells. The initial blastodermal cells present a germ line competency, but their *in vitro* plating and long term cultures lead to a progressive loss of this property. Similar observation was carried out for the primordial germ cells (PGCs) taken either from embryonic blood or already present in the developing gonads. The resulting EG cells no longer harbor their parental germline competency. Only PGCs maintained under specific non-adherent culture conditions were demonstrated to retain this property. By following the level of expression of *Cvh* and *Dazl* genes in these different cell types, a clear correlation can be sent between the expression of these two genes and the ability of the cells to colonize the gonads and to differentiate into functional gametes. We are therefore proposing to use these markers as germ line sensors to control and follow the germ line competency of the different isolated pluripotent chicken stem cells.

petency through an active role of the *Cvh* gene. The active development of molecular tools, presently available in chicken, will allow us and others to propose in the coming months a better view of the gene network associated with the maintenance of pluripotency and germline competency in chicken. These data will also open the way for various new investigations including the identification of putative chicken EpiSCs and the demonstration of the presently hypothetical existence of a reprogramming process in avian species.

We are indeed convinced that cESCs represent a unique model to study and to bring a better understanding of stem cell physiology in non-mammalian species. For all of these reasons we are still developing and investigating this valuable model and these remarkable cells.

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Conflict of interest

No conflict of interest has been declared.

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