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Evolutionary view of pluripotency seen from early development of non-mammalian amniotes

Shota Nakanoh [a,b,c,**], Kiyokazu Agata [d,*]

[a] Division of Embryology, National Institute for Basic Biology, Okazaki 444-8787, Japan
[b] Wellcome–MRC Cambridge Stem Cell Institute, Anne McLaren Laboratory, University of Cambridge, Cambridge CB2 0SZ, UK
[c] Department of Surgery, University of Cambridge, Cambridge CB2 0QQ, UK
[d] Graduate Course in Life Science, Gakushuin University, Toyoshima-ku, Tokyo 171-8588, Japan

A B S T R A C T

Early embryonic cells are capable of acquiring numerous developmental fates until they become irreversibly committed to specific lineages depending on intrinsic determinants and/or regional interactions. From fertilization to gastrulation, such pluripotent cells first increase in number and then turn to undergoing differentiation. Mechanisms regulating pluripotency in each species attract great interest in developmental biology. Also, outlining the evolutionary background of pluripotency can enhance our understanding of mammalian pluripotency and provide a broader view of early development of vertebrates. Here, we introduce integrative models of pluripotent states in amniotes (mammals, birds and reptiles) to offer a comprehensive overview of widely accepted knowledge about mammalian pluripotency and our recent findings in non-mammalian amniotes, such as chicken and gecko. In particular, we describe 1) the IL6/Stat3 signaling pathway as a positive regulator of naive pluripotency, 2) Fgf/Erk signaling as a process that prepares cells for differentiation, 3) the role of the interactions between these two signaling pathways during the transition from pluripotency to differentiation, and 4) functional diversification of two transcription factors, Class V POUs and Nanog. In the last section, we also briefly discuss possible relationships of unique cell cycle properties of early embryonic cells with signaling pathways and developmental potentials in the pluripotent cell states.

1. Introduction

Early development of vertebrates features cells that are flexible regarding their fate decision. Before gastrulation, mammalian embryos experience a series of developmental events: segregation of the inner cell mass and trophectoderm, formation of the epiblast and hypoblast, and implantation (Fig. 1A). Shortly after E-cadherin-dependent compaction and cavitation (Shirayoshi et al., 1983), a fertilized egg develops to a blastocyst by segregating two clusters of cells – the inner cell mass (ICM) and the trophectoderm (TE). In the late blastocyst stage, cells in the ICM separate into epiblast and hypoblast, while the TE forms a part of the placenta to support the fetal body. The epiblast is the group of pluripotent cells that further develop into the embryo proper. The hypoblast (also called “primitive endoderm” in mouse studies) is the extraembryonic lineage beneath the epiblast layer and provides the epiblast cells with positional information and differentiation cues. Around the completion of epiblast and hypoblast formation, mammalian embryos attach to the maternal uterus. This is called implantation, and is followed by gastrulation.

During these developmental events in mammalian pre-gastrula development, the uncommitted cells which decided not to follow the extraembryonic lineages go through two distinct states of pluripotency – naive state and primed state (Fig. 1A). Naive pluripotency is the state of the cells in the ICM or early epiblast before implantation, whereas primed pluripotency is the state of the cells in the late epiblast in already implanted embryos. Through the transition between these states, the uncommitted cells become ready for the differentiation that will occur upon gastrulation, while retaining pluripotency (Nichols and Smith, 2009; Smith, 2017). Naive and primed pluripotency have different features, such as signal requirements to support the state, while sharing core pluripotency transcription factors. In the mouse naive state, LIF/Stat3 signaling and downstream transcription factors are known to act as the maintaining factors, while Fgf/Erk and Gsk3β trigger the cells to exit from the naive pluripotency (Fig. 1B) (Wray et al., 2011). In contrast, primed pluripotency is supported by Fgf/Erk and Tgfβ/Smad signaling pathways (Vallier, 2005).

Although ICM/TE segregation and implantation seem to be mammalian-specific events, epiblast and hypoblast formation is commonly seen among amniotes. Indeed, the basic frameworks of development appear to be quite similar in mammals and non-mammalian

* Corresponding author.
** Corresponding author. Division of Embryology, National Institute for Basic Biology, Okazaki 444-8787, Japan.
E-mail addresses: sn510@cam.ac.uk (S. Nakanoh), kiyokazu.agata@gakushuin.ac.jp (K. Agata).

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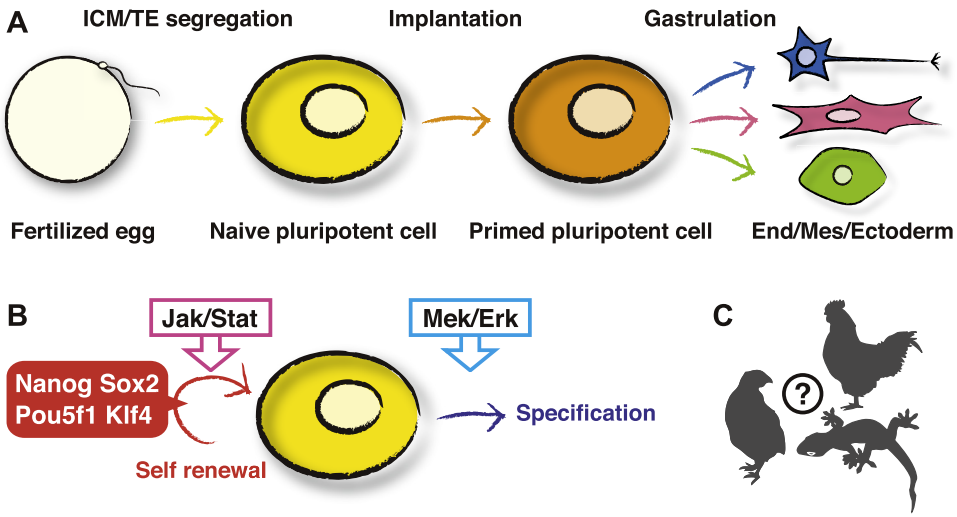

Fig. 1. Overview of the early development and the mechanisms of pluripotency in mammals. (A) Transition of mammalian embryonic cells from fertilization until gastrulation. Developmental events occur around the transitions (arrows) but do not necessarily drive the transitions. (B) Positive and negative regulators of mouse naive pluripotency. (C) In this review, we ask which components of the mechanisms underlying mammalian pluripotency also work in avian and reptile pluripotent cells.

amniotes – embryos have the flexibility to alter their fate maps until gastrulation. Chicken embryos can change the cellular fate to regenerate the surgically removed parts and form complete miniature embryos from dissected pieces until gastrulation starts at Hamburger and Hamilton stage 4 (HH 4) (Bertocchini et al., 2004; Hamburger and Hamilton, 1951; Spratt and Haas, 1960), although the regulative ability of reptile embryos is not clearly understood because of the limited availability of the embryos. To what extent are the mechanisms underlying pluripotency conserved among mammals, birds and reptiles? What is the ancestral form of regulation of pluripotency in amniotes? (Fig. 1C).

Here, we aim to revisit the mechanisms of mammalian pluripotency in the context of development and evolution, and to provide possible integrative models for early development of amniotes, based on our recent publications and findings from unpublished data regarding non-mammalian species, mainly chicken.

2. Conserved role of IL6/Stat3 signaling to support naive pluripotency in the early epiblasts

Until gastrulation, when cells become committed to three germ layers, epiblast cells are required to proliferate while remaining uncommitted to any of the particular fates. What mechanisms support this cell division?

Establishment of mouse embryonic stem cells (ESCs) provided a useful tool to study the mechanisms of pluripotency *in vitro* (Evans and Kaufman, 1981; Martin, 1981). At first, fibroblasts were used as the feeder cells to keep mouse ESCs in the pluripotent state, and later LIF (leukemia inhibitory factor) was demonstrated to be the responsible factor expressed and provided by the feeders (Smith et al., 1988; Williams et al., 1988). LIF belongs to the IL6 (interleukin 6) family, whose members generally act as ligands to activate the Jak/Stat3 (Janus kinase/Signal transducer and activator of transcription 3) signaling pathway (Hirano et al., 2000). Moreover, although they do not express IL6 receptor, mouse ESCs can also be maintained by supplying IL6 and soluble IL6 receptor in culture (Nichols et al., 1994). Following the discovery of the contribution of the IL6 family to maintaining mouse ESCs, Jak/Stat3 signaling was identified as the key pathway downstream to these ligands (Matsuda, 1999; Niwa et al., 1998). However, the fact that mouse embryos genetically deficient for *LIF*, *LIF receptor*, *gp130* (common subunit of the IL6-family receptors) or even *Stat3* can still survive at least until the primitive streak stage, cast some doubt on the functional role of IL6/Stat3 signaling to support pluripotent cells during mouse early development *in vivo* (Nichols et al., 2001; Takeda et al., 1997). In addition, human pluripotent cell lines were established and maintained independently of IL6/Stat3 signaling (Thomson, 1998; Vallier, 2005), raising the possibility that a Stat3-dependent pluripotent state of early embryonic development may not be a common feature even in mammals.

The confusion about the necessity for IL6/Stat3 signaling for pluripotency was resolved in 2013, when Do et al. reported that Stat3 signaling becomes active from the 4-cell stage onward in mouse early embryos, and LIF and IL6 are responsible for this activation. When Stat3 was eliminated from both oocytes and zygotes, mouse embryos could not expand the pluripotent epiblast, suggesting that Stat3 (either stored in the oocyte or expressed in the zygote) is necessary for the growth of the mouse pluripotent cell population even during normal development (Do et al., 2013). Also, *in vitro* cultures of human pluripotent cells dependent on the Stat3 signaling pathway have been established recently (Chen et al., 2015; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Zimmerlin et al., 2016). These new findings suggest that IL6/Stat3 signaling has the function of supporting undifferentiated cells during the normal processes of early development in a wide range of mammalian species.

In spite of this growing evidence that IL6/Stat3 signaling forms an axis to positively regulate pluripotency in normal mammalian development, its function during early development of non-mammalian species remained unclear. Recently, we found that pluripotent cells are similarly supported by IL6/Stat3 signaling in chicken embryos (Nakanoh et al., 2017). We analysed transcriptional dynamics of developing chicken embryos in search of the genes highly expressed before HH 4, when cells start to differentiate, and found that transcripts of *IL6* and *Socs3* (suppressor of cytokine signaling 3, a downstream target giving negative feedback to Stat3 signaling) were significantly more abundant before than after gastrulation. Immunostaining revealed that nuclear localization of active Stat3 protein was only found before gastrulation, suggesting that the IL6/Stat3 signaling pathway is specifically active before chicken embryonic cells start to differentiate (Nakanoh et al., 2017). Transcripts of *IL6* and *Socs3*, and active Stat3 were lost quickly upon gastrulation, indicating that the activity of this signaling pathway is strictly regulated before and after gastrulation (Fig. 2A).

We also developed an *in vitro* system to assay the effects of signaling pathways on chicken embryonic cells (Nakanoh et al., 2015, 2013). In the neutral culture condition, chicken blastodermal cells form densely packed colonies with distinct expression of Nanog, which is a reliable marker of chicken pluripotent cells and primordial germ cells (see section 4). However, when cultured under Jak1 inhibition, these colonies deteriorated and lost Nanog expression. This effect of the Jak1 inhibitor was counteracted when active Stat3 was artificially induced by exogenous gene induction. Taken together, these results suggest that IL6/Stat3 signaling mediated through Jak1 functions to support pluripotency via the maintenance of Nanog expression during chicken early development

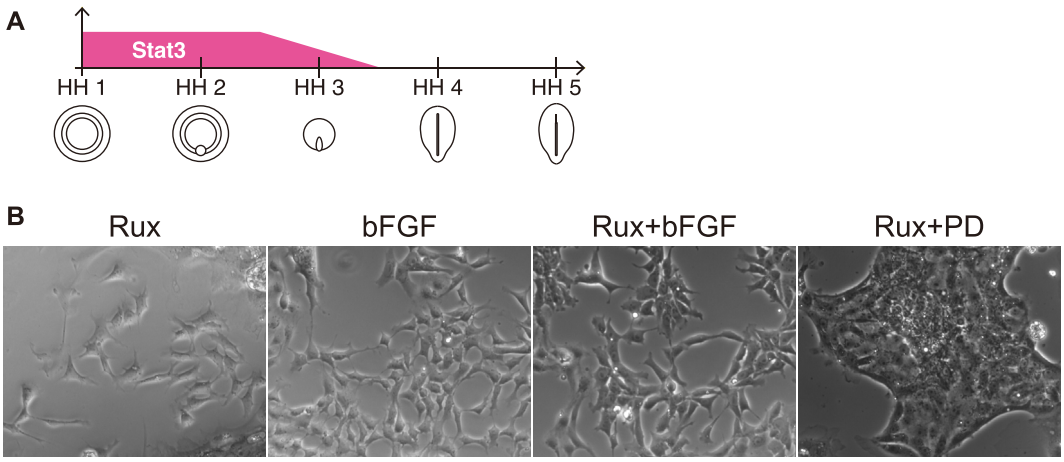

Fig. 2. Roles of the IL6/Stat3 signaling pathway during chicken early development. (A) Activity of IL6/Stat3 signaling during chicken development suggested by the transcriptome analysis and the immunohistochemistry study. Lower schematic drawings show chicken embryos at the stages. HH 1 is the stage when embryos form blastoderms containing pluripotent epiblast and extraembryonic hypoblast. HH 4 is when gastrulation takes place and epiblast cells determine their fate irreversibly. (B) Resemblance of cells appearing under IL6/Stat3 inhibition and Fgf/Erk activation. When bFgf was added to the Rux condition, more of these cells were observed. In contrast, supplement of Mek inhibitor addition to Rux strongly suppressed these dispersed cells. Rux: Ruxolitinib (Jak1/2 inhibitor), PD: PD0325901 (Mek inhibitor). Photos are at the same magnification.

(Nakanoh et al., 2017). Interestingly, the IL6/Stat3 signaling appeared to be dispensable for the cells to differentiate into three germ layers. Even under Jak/Stat3 inhibition, there were vigorously proliferating populations of Nanog-negative cells (Fig. 2B). These cells were morphologically similar to the ones observed in the culture with Fgf2 (Fig. 2B) (Nakanoh et al., 2013). Also, in the *ex ovo* culture, chicken blastoderms developed primitive streaks normally even when Stat3 signaling was inhibited (Nakanoh et al., 2017). These findings suggest that IL6/Stat3 signaling is important for the undifferentiated cells to survive and proliferate, but becomes dispensable once the cells proceed to the advanced phase of commitment. Intriguingly, Stat3 activated by the Wnt/β-catenin pathway is required for cell migration but not for cell fate determination during gastrulation in zebrafish (Yamashita et al., 2002). Thus, the general function of IL6/Stat3 signaling in pre-gastrula embryos might be to support fundamental cell properties, such as proliferation, without inducing specification to any particular lineages in uncommitted cells.

An extended stage of development called embryonic diapause has been thought to explain the discrepancy between *in vitro* and *in vivo* observations on requirements for IL6/Stat3 signaling (Nichols et al., 2001). In mouse, embryonic diapause is induced by the low-estrogen conditions caused when the mother experiences lactation or ovariectomy (Hondo and Stewart, 2005; Mantalenakis and Ketchel, 1966). Mouse embryos in diapause develop to and stop at the blastocyst stage and retain their developmental potential without undergoing implantation to uteri. IL6/Stat3 signaling is necessary for the mouse epiblast cells to maintain pluripotency during diapause, as shown by the fact that embryos genetically deficient for gp130 were not able to resume development after diapause. Therefore, the culturability of mouse ESCs enabled by LIF *in vitro* was considered to be a reflection of the ability of the mouse embryos to survive diapause *in vivo* (Nichols et al., 2001). However, two recent publications revealed that the Myc pathway and the mTOR (mechanistic target of rapamycin) pathway regulate the entry to and exit from the diapause state in mouse blastocysts (Bulut-Karslioglu et al., 2016; Scognamiglio et al., 2016). Importantly, these papers demonstrated that the inhibition of either the Myc pathway or the mTOR pathway in mouse ESCs induced reversible reduction of transcription, translation and proliferation without affecting pluripotency, as seen in embryonic diapause. Furthermore, the transcription signatures of the hormonally diapaused epiblasts were closer to those of these dormant mouse ESCs than of the non-treated mouse ESCs, suggesting that mouse ESCs maintained by LIF with normal levels of Myc and mTOR activities are different from the cells in diapause embryos.

Our findings from chicken embryos together with the aforementioned observations in mammalian models suggest that the pathway from IL6 ligands to Jak/Stat3 signaling acts as the positive regulator of the naive state of pluripotency during normal development of amniotes. In order to supply a sufficient number of undifferentiated cells before gastrulation, the amniotes' early epiblasts in normal development would use IL6/Stat3 signaling to support the cell division and survival without initiating differentiation. This might be the reason why we are able to derive Stat3-dependent pluripotent stem cell cultures from various species, including rat and chicken (Buehr et al., 2008; Li et al., 2008), although systems tuned up for diapause could further stabilize the IL6/Stat3-dependent state in some particular species or strains permissive for the derivation of naive pluripotent cell lines. Alternatively, it is also possible that IL6/Stat3-dependent diapause could be a common feature among these species, and early processes of normal development might have evolved to utilize it. Embryonic dormancy is widely seen among species, and avian embryos can resume early development that has once been paused by low temperature (Fasenko, 2007).

3. Conserved role of Fgf/Erk signaling to prepare epiblast cells for differentiation into three germ layers

We have seen that IL6/Stat3 signaling appears to be a common positive regulator of the pluripotency in early naive epiblasts of amniotes. Then, what directs the cells toward differentiation into three germ layers upon gastrulation?

Fgf (fibroblast growth factor) signaling is known to be a critical element for gastrulation, especially mesendoderm lineage formation, in various kinds of species (Dorey and Amaya, 2010). Erk (extracellular signal-regulated kinase) is a classical Mapk (mitogen-activated protein kinase) acting as one of the major downstream transducers of Fgf signaling, and is activated through Mek (Mapk/Erk kinase) (Thisse and Thisse, 2005). Fgf/Erk signaling act as a multifunctional regulator of mammalian pluripotency to drive cells towards differentiation. More specifically, Fgf/Erk signaling appears to facilitate the cells' 1) exit from naive pluripotency, 2) establishment and maintenance of primed pluripotency and 3) entry into one of the three germ layers.

In mouse and human, there are two Erk genes responsible for this pathway: Erk1 (Mapk3) and Erk2 (corresponding to both Mapk1 and Mapk2) (Boulton et al., 1991). According to Frémin et al. (2015), Erk2 plays major roles during mouse development, although the total activity of the two isozymes is deterministic. Consistent with this, Erk1-KO mice

are viable, fertile and of normal size (Pages et al., 1999), while Erk2-KO mice are not able to develop mesendodermal tissues and die around gastrulation (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). *In vitro* studies have suggested that Fgf/Erk signaling is necessary for the transition from the naive pluripotent state to the primed pluripotent state because activation of Fgf/Erk signaling causes transition from the naive state to the primed state, while its inhibition conversely stabilizes the naive state (Greber et al., 2010; Guo et al., 2009). When Fgf/Erk signaling is disturbed pharmacologically or genetically, mouse ESCs cannot differentiate into neuroectoderm or mesendoderm lineages (Kunath et al., 2007; Stavridis et al., 2007). In contrast, primed pluripotent cells, such as mouse epiblast stem cells (EpiSCs) and conventional human ESCs, maintain their developmental competency though activation of Fgf/Erk signaling and Tgfβ/Smad (transforming growth factor β) signaling (Brons et al., 2007; Tesar et al., 2007; Vallier, 2005). These facts suggest that Fgf/Erk signaling is important for exit from the naive state to the primed state, and for maintenance of the primed state.

In chicken, only one Erk gene has been identified so far. When Fgf/Erk signaling was pharmacologically blocked, chicken blastoderms were not able to produce Brachyury-positive mesendodermal precursor cells, and the gastrulation was severely disrupted (Fig. 3A and B) (Bertocchini et al., 2004). Also, initiation of neuronal lineage specification was blocked by the inhibition of Fgf/Erk signaling at the ectoderm region in gastrulating chicken embryos (Stavridis et al., 2007). In addition to these *in vivo* observations, our cell culture-based studies suggested that Fgf/Erk signaling also prepares avian and reptile pluripotent cells for differentiation (Nakanoh et al., 2013, 2015), although it is still not clear if pluripotency equivalent to the mammalian primed state exists in avian and reptile development. When chicken blastodermal cells were cultured in a Mek inhibitory condition, they formed Nanog-positive colonies which were morphologically similar to the ones formed by naive mouse or human ESCs. These dome-shaped colonies were formed only from pre-gastrula embryos, which retain pluripotent cells, but not from post-gastrula embryos. In contrast, when the blastodermal cells were cultured instead in Fgf2-containing medium, they swiftly lost Nanog expression and generated flattened colonies with migrating cells (Figs. 2B and 3C, also see Nakanoh et al., 2013). Similar responses were observed with quail, gecko and turtle embryonic cells (Nakanoh et al., 2013). Interestingly, planarian flatworms also utilize Fgf/Erk signaling to induce differentiation of somatic pluripotent stem cells when regenerating (Tasaki et al., 2011; Umesono et al., 2013). Taken together, these findings from various species suggest that Fgf/Erk signaling is generally required to drive naive pluripotent cells towards differentiation into the three germ layers.

Although the functions of the signaling pathways seem to be conserved among amniotes, we have not succeeded in maintaining cultures of avian and reptile naive pluripotent cells by simply activating IL6/Stat3 signaling and/or inhibiting Fgf/Erk signaling in serum-free medium (Nakanoh et al., 2013, 2017). This may suggest that the cell cycle progression was not sufficiently induced by controlling these signaling pathways in the culture. Considering this possibility, activating cell cycle promoters, such as Myc genes, as well as regulating the signaling pathways is worth a try in order to establish proliferative pluripotent cell lines derived from non-mammalian species.

4. Interactions between IL6/Stat3 and Fgf/Erk signaling pathways during the transition from pluripotent to differentiating state

As shown in section 1, the activity of IL6/Stat3 signaling is specific for the early blastoderms and swiftly decreases upon gastrulation, implying the existence of specific mechanisms that shut down this signaling pathway in chicken embryos (Fig. 2A). In contrast, both the phosphorylation of Erk and the transcript levels of the molecular components of the Fgf/Erk signaling pathway, such as Fgf receptors and Mkp3 (mitogen-activated protein kinase phosphatase 3, a downstream target giving negative feedback to Erk signaling) increase at the gastrulation stage, suggesting that this signaling pathway becomes active upon gastrulation (Fig. 3D) (Lunn et al., 2007). Therefore, the activity of IL6/Stat3 signaling and that of Fgf/Erk signaling appear to be mutually exclusive during chicken early embryogenesis. Studies in blood cells revealed that phosphorylated Erk inactivates Stat3 protein by direct protein-protein interactions (Chung et al., 1997; Sengupta et al., 1998). Hence, it is reasonable to suppose that the increase of Fgf/Erk signaling directly attenuates IL6/Stat3 signaling in chicken gastrulas at around HH 4.

Additionally, we found that the IL6/Stat3 and Fgf/Erk signaling pathways exert mutual inhibitions on each other and form positive feedbacks on themselves by regulating the expression levels of their ligands. We measured the transcript levels of *IL6* and *Fgf*s in chicken embryos cultured *ex ovo* for 24 h (Fig. 4A). Compared with the control DMSO condition, the expression level of *IL6* was dramatically elevated by the sole inhibition of Mek (70 times higher than in the control embryos), but this elevation was blocked by the co-inhibition of Jak and Mek. Expression of *Fgf4*, a gastrulation-related *Fgf* in chicken development

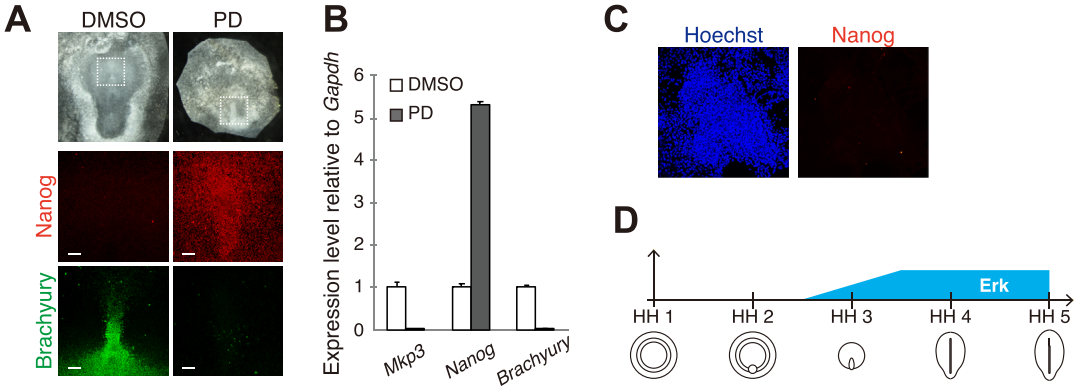

Fig. 3. Roles of the Fgf/Erk signaling pathway during chicken early development. (A) Whole-mount immunohistochemistry of chicken embryos after 24-h *ex ovo* culture. Embryos were co-stained for Nanog and Brachyury. Boxed regions in the bright field view are magnified below. Scale bars: 100 μm. (B) Gene expression analysis following the *ex ovo* culturing in the indicated conditions. Severe downregulation of Mkp3 shows that the Mek inhibition is working in this system. Bars show mean ± s.d. from replicate qRT-PCR assays (n = 3) of pooled RNA from three embryos. DMSO: dimethyl sulfoxide (control), PD: PD0325901 (Mek inhibitor). (C) Immunohistochemistry of chicken blastodermal cells cultured with bFgf *in vitro*. Colonies were flattened and swiftly lost Nanog expression. (D) Activity of the Fgf/Erk signaling pathway in chicken development suggested by transcription levels of upstream and downstream factors and phosphorylation of Erk proteins in the previous literature. They rise around HH 4, but are low in the pre-gastrula embryos.

(Hardy et al., 2011), was clearly decreased by the Mek inhibitor and not obviously influenced by the Jak inhibitor. *Fgf5* was not so much affected by the inhibitors, which is consistent with its weak expression in the chicken gastrula (Kumar and Chapman, 2012). Interestingly, *Fgf8*, another *Fgf* member important for gastrulation (Lawson et al., 2001), showed increased expression when Jak1/Stat3 signaling was blocked, and this upregulation was not observed in the presence of both the Jak and Mek inhibitors. Taken together, these results suggest that the IL6/Stat3 and Fgf/Erk pathways have positive feedbacks on themselves by upregulating their own ligands. It is also suggested that IL6/Stat3 signaling antagonizes Fgf/Erk signaling by downregulating *Fgf8* transcription, while Fgf/Erk signaling in turn suppresses the expression of *IL6* (Fig. 4B). Although their interactions are bidirectional, the suppression of *IL6* expression by Erk signaling was far stronger than the suppression of *Fgf8* by Stat3 signaling. In addition, IL6 signaling itself can stimulate the Fgf/Erk feedback loop because the receptor tyrosine kinase activity of IL6 family members activates Erk signaling in parallel to Stat3 signaling (Hirano et al., 2000). Together with the direct protein-protein interaction between Stat3 and Erk, these mechanisms may guarantee that the balance between these two signaling pathways comes to favor the Fgf/Erk side such that gastrulation and differentiation proceed (Fig. 4B). It might be the case that the conserved role of Fgf/Erk signaling during early embryogenesis is to terminate the IL6/Stat3 signaling.

Transcriptome analyses revealed that the mutually exclusive expression patterns of Stat3 signal components, such as *LIF receptor* and *Stat3* and Erk signal components, such as *Fgf4* and *Fgf5* can also be seen during mouse early development (Boroviak et al., 2015; Kalkan et al., 2017). Intriguingly, in mouse ESCs, supplement of LIF leads to the downregulation of *Fgf5*, which is expressed restrictedly in the cells committing to the three primary germ layers during mouse gastrulation (Hébert et al., 1991; Kunath et al., 2007). Also, gastrulation and mesendoderm formation were prevented in mouse embryos overexpressing LIF (Conquet et al., 1992). Collectively, the abovementioned results suggest that mutual interactions between the Stat3 and Erk signaling pathways could also function in mouse development. Based on these observations, we propose a model of amniotes' early development in which epiblast cells transit from a Stat3-dependent state to an Erk-dependent state while blastula develops into gastrula (Fig. 4C). Along with this shift of signal pathways, epiblast cells pass from the naive state to the differentiating state through a primed state and become ready for the differentiation into three germ layers. Also, cell division and survival appear to be dependent on each signaling pathway (discussed in section 5).

5. Diversified transcription networks regulating pluripotency

We have seen that signaling pathways, such as IL6/Stat3 and Fgf/Erk appear to have conserved roles among amniotes in regulating pluripotency. However, in mammals, pluripotency-associated transcription factors are thought to be essential because they can reprogram the somatic cells into pluripotent states (Takahashi and Yamanaka, 2006). To what extent are these transcription factors conserved among amniotes?

In mammals, Pou5f1 (also known as Oct3/4) is the pivotal pluripotency factor for both naive and primed states. Pou5f1 protein binds to other pluripotency transcription factors and genomic regions to function as a transcriptional core regulator of pluripotency (Pardo et al., 2010). Loss of functional Pou5f1 by gene disruption or RNA interference leads to severe defects of the maintenance of pluripotency (Nichols et al., 1998; Niwa et al., 2000; Okamoto et al., 1990; SchOler et al., 1990), and the efficiency of the reprogramming of somatic cells to a pluripotent state becomes quite low when *Pou5f1* is removed from the transgene cocktail (Takahashi and Yamanaka, 2006). Importantly, Pou5f1 acts to specify the pluripotent ICM population instead of the extraembryonic TE population by inhibiting Cdx2, which drives cells to the TE lineage (Niwa et al., 2005). The involvement of Pou5f1 in this mammalian-specific developmental event suggests that the function of Pou5f1 as the key pluripotency transcription factor might also be unique to mammals. Indeed, birds and some orders of reptiles do not have *Pou5f1* but have a similar, distinct paralogous gene, *Pou5f3* (Frankenberg et al., 2014; Nakanoh et al., 2015; Niwa et al., 2008), supporting this hypothesis.

Pou5f3 is the member of the Class V POU family closest to Pou5f1 in vertebrates. Based on their syntenies, *Pou5f1* and *Pou5f3* are thought to have been generated by a multigenic duplication which occurred before the separation of cartilaginous and teleost fish (Frankenberg and Renfree, 2013). Interestingly, losses of the *Pou5f1* or *Pou5f3* gene have independently occurred multiple times during vertebrate evolution. As a result, eutherian mammals and squamates have *Pou5f1* but not *Pou5f3*, while birds, crocodiles, frogs and teleost fish have only *Pou5f3* instead (Frankenberg et al., 2014). Complementation assays of the Class V POU genes revealed that *Pou5f3* orthologues from most species cannot enable long-term maintenance of mouse ESCs deficient for endogenous *Pou5f1*

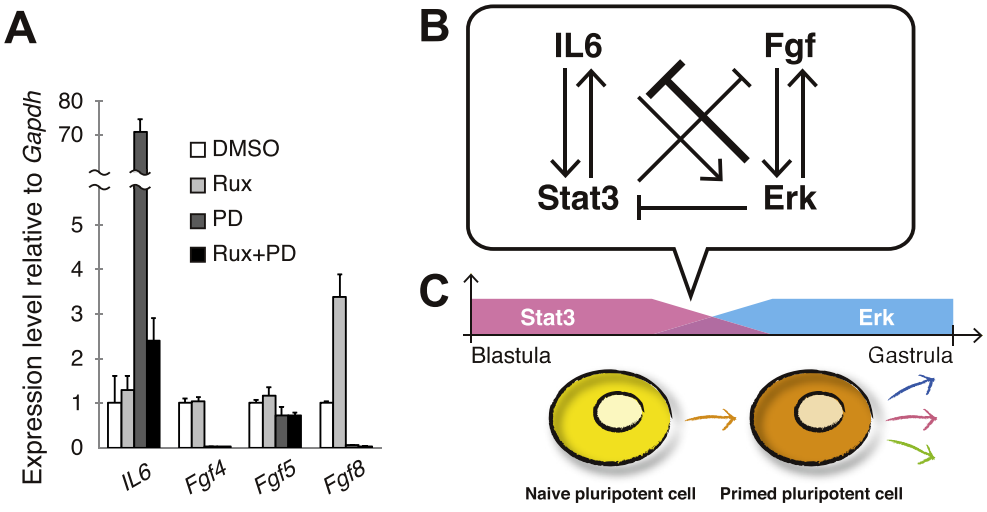

Fig. 4. Interactions between IL6/Stat3 signaling and Fgf/Erk signaling through regulation of their ligands. (A) Gene expression analysis following 24-h *ex ovo* cultures in the indicated conditions. Bars show mean ± s.d. from replicate qRT-PCR assays (n = 3) of pooled RNA from three embryos. DMSO: dimethyl sulfoxide (control), Rux: Ruxolitinib (Jak1/2 inhibitor), PD: PD0325901 (Mek inhibitor), Rux + PD: combination of Rux and PD (co-inhibition). (B) Summary of interactions between the IL6/Stat3 and Fgf/Erk signaling pathways. Direct inhibitions of Stat3 by Erk and simultaneous activation of Erk by IL6 are taken from the literature. (C) A model describing the transition during early development of amniotes. Pluripotent epiblast cells become ready for differentiation upon gastrulation by passing through a Stat3-dependent naive pluripotency and an Erk-dependent primed pluripotency. The interaction between IL6/Stat3 signaling and Fgf/Erk signaling (B) may have a role to smoothly switch undifferentiated states to differentiating states.

(Lavial et al., 2007; Morrison, 2006; Niwa et al., 2008), suggesting that these paralogues may be functionally diversified despite their sequence similarities. Class V POU factors consist of three regions: the N-terminal domain, POU domain and C-terminal domain. The POU domain, which consists of a POU-specific domain and a POU homeodomain connected by a linker region, is responsible for the DNA binding, while the N- and C-terminal domains are thought to act as transactivation domains that function by interacting with other trans-factors (Imagawa et al., 1991; Niwa et al., 2002). Alignments of amino acid sequences showed that Pou5f1 and Pou5f3 are quite diverse at the N-terminal and C-terminal regions while highly conserved at the POU domain (Frankenberg et al., 2010; Lavial et al., 2007; Morrison, 2006; Nakanoh et al., 2015). It is possible that the multiple independent deletions of *Pou5f1* and *Pou5f3* that occurred during vertebrate evolution happened because these similar but different genes could be not just redundant but actually toxic. Pou5f1 and Pou5f3 may compete on similar genomic targets while recruiting different factors when expressed together in a cell.

Nanog is another central pluripotency transcription factor, and was first identified as a downstream factor of LIF signaling in mouse ESCs (Chambers et al., 2003; Mitsui et al., 2003). In mammalian embryos, Nanog is exclusively expressed in pluripotent epiblast cells and primordial germ cells, and is necessary to form the epiblast cell population (Chambers et al., 2003; Mitsui et al., 2003). Nanog forms a reciprocal inhibitory circuit with Gata4 and Gata6, which direct cells in the ICM to the hypoblast lineage (Frankenberg et al., 2011). In contrast to the mammalian-specific segregation of the ICM and TE, formation of the epiblast and hypoblast is commonly observed in amniotes (Stern and Downs, 2012). Also, in chicken embryogenesis, Nanog is first expressed widely in the pluripotent blastoderm (equivalent to the mammalian blastocyst) and then localized in epithelialized epiblasts (Lavial et al., 2007; Nakanoh et al., 2015), implying that Nanog plays similar roles to maintain the pluripotent population and suppress hypoblast-specifying genes in birds and reptiles. Complementation assays showed that Nanog orthologues from chicken and zebrafish could bind to and regulate the downstream targets when mouse somatic cells deficient for endogenous Nanog were reprogrammed. Furthermore, the resulting iPSCs (induced pluripotent stem cells) could be maintained without LIF in culture (Theunissen et al., 2011). These results suggest that Nanog orthologues of vertebrate species may possess conserved functions.

In addition to inducing the epiblast fate and suppressing the hypoblast fate, it has been suggested that Nanog is essential in generating the naive state of mammalian pluripotency, and that overexpression of Nanog facilitates primed cells to enter into the naive state (Okita et al., 2007; Silva et al., 2009; Takashima et al., 2014). In mouse development, Nanog is quickly downregulated upon implantation (embryonic day 4.7–5.0) and is regained until embryonic day 8.0 (Acampora et al., 2012; Hatano et al., 2005). This rapid and temporary disappearance of Nanog expression, which indicates the collapse of naive pluripotency network and is related to lumenogenesis in the post-implantation embryos (Shahbazi et al., 2017), has not been observed in monkey or human embryos (Nakamura et al., 2016; Shahbazi et al., 2017). Chicken Nanog also appeared to be continuously expressed until becoming undetectable in the gastrulating mesendodermal cells and specified ectodermal cells (Lavial et al., 2007; Nakanoh et al., 2015). Human and chicken embryos form morphologically similar disk-shaped epiblasts, while mouse epiblast develops in a cup-shaped structure. However, human embryos form the amniotic cavity enclosed by the epiblast and amniotic epithelium at the pre-gastrula stage (Rossant and Tam, 2017). In contrast, the chicken pluripotent epiblast lies just beneath the vitelline membrane having no obvious amniotic cavity, although a distinct space called subgerminal cavity is formed between the epiblast and yolk at the cleavage stage (Hamburger and Hamilton, 1951; Kochav et al., 1980). It is interesting to investigate whether or not these morphological differences and similarities influence molecular pathways in fate decisions.

Considering the functional conservation of Pou5f1 and Nanog in regulating pluripotency and the developmental events they govern, we provide a model describing the embryonic/extraembryonic lineage segregations during early development in amniotes (Fig. 5A). In this model, birds represent the default form of the early development in which epiblast and hypoblast are formed under the control of Nanog without ICM/TE segregation, while mammalian ICM/TE formation is interpreted to be added to this basic form. Recent establishment of mammalian pluripotent cell lines able to differentiate into both ICM and TE lineages (Yang et al., 2017) might provide unique opportunities to seek an event equivalent to mammalian ICM/TE segregation and the molecular mechanisms underlying it in non-mammalian amniotes. The lack of Pou5f1, the hub transcription factor, in some species suggests that the pluripotency network could be diversified among these species. Considering this possibility together with the conserved roles of signaling pathways discussed in the earlier sections, we propose that the signaling pathways might be the unchangeable core part while their downstream transcription networks could be modifiable in order to achieve pluripotency in divergent forms of early development in each species (Fig. 5B) (Nakanoh et al., 2017).

6. Possible relationships among cellular potential, signaling pathway and cell division during early development

As we saw in section 3, early epiblast cells transit from the IL6/Stat3-dependent state to the Fgf/Erk-dependent state (Fig. 4C). In chicken, cells derived from embryos that have not yet gone through gastrulation suffer severely under the suppression of IL6/Stat3 signaling, while embryos are able to develop to gastrulas even when IL6/Stat3 signaling is blocked *in vivo*, suggesting that this signaling pathway is no longer necessary for the cells that are differentiating quickly (Nakanoh et al., 2017). This is consistent with the finding that the cells morphologically similar to those in the Fgf condition proliferated actively under Jak inhibition (Fig. 2B). In clear contrast, cells derived from the early epiblast survive and proliferate rather well in the presence of the Mek inhibitor, while cells collected from more advanced embryos already initiating gastrulation cannot propagate in its presence (Nakanoh et al., 2013). *Ex ovo* culture revealed that embryos cannot proceed to gastrulation but remain Nanog-positive when Fgf/Erk signaling is blocked (Fig. 4A). Low dependence on Erk signaling in naive pluripotency has been well demonstrated in mammals by chemical and genetic perturbation (Nichols et al., 2009; Takashima et al., 2014; Theunissen et al., 2014; Ying et al., 2008). Erk is essential for cell division and survival in various types of cells, and contributes to driving the cell cycle mainly by promoting G1/S transition through cyclin D1 regulation (Chambard et al., 2007). Therefore, the Erk-independence of the early epiblast cells appears to be a unique but conserved feature of early pluripotent Stat3-dependent proliferating cells.

In general, ontogeny begins with cleavage, which corresponds to rapid cell division with short G1 and G2 phases, and then cells extend their gap phases while undergoing lineage commitment during development. We have found that chicken embryonic cells reduce in volume toward HH 4, suggesting that these early embryonic cells keep the cleavage-like short cell cycles until they become differentiated (data not shown). Consistently, both mouse and human ESCs are reported to run short gap phases while they are pluripotent (Becker et al., 2006; White and Dalton, 2005). Pauklin and Vallier revealed that G1 Cdks and cyclins regulate the sensitivity to Tgfβ/Smad signaling in human ESCs, suggesting that cell cycle machineries can act upstream of signaling pathways to regulate the cellular potentials (Pauklin and Vallier, 2013). Considering the coincidence of the changes in cell signaling, cellular potential and cell cycle during the pre-gastrula development of amniotes, it is interesting to ask what are the molecular links between the cleavage-like cell division and IL6/Stat3 and Fgf/Erk signaling pathways. Further studies are awaited to examine how signal requirements, reconstruction of cell cycle profiles and cellular potentials are related in the early embryonic pluripotent cells.

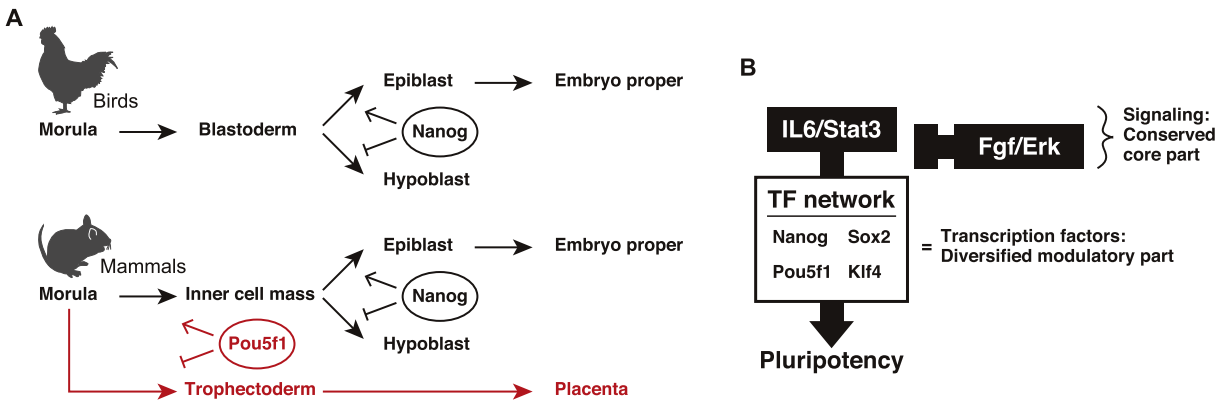

Fig. 5. (A) A model describing the binary determinations of cell fates during the early developmental events in amniotes. We propose that segregation of the epiblast and hypoblast in blastocysts conducted by Nanog is the default developmental path (shown in black) as represented in birds, while mammals have this default path and an additional TE lineage formation dictated by Pou5f1 (highlighted in red). (B) A schematic diagram showing the conserved and diversified parts of molecular components regulating pluripotency of amniotes. It was suggested that signaling pathways have conserved roles while the downstream transcription networks are more modifiable among species.

7. Conclusion

In this review, we revisited the mechanisms of pluripotency described in mammals along with our findings about embryos of non-mammalian amniotes, and provided models integrating the early development of mammals, birds and reptiles. Some molecular components underlying mammalian pluripotency appear to be common, while others appear to be diversified, among these species (Fig. 5B). Signaling pathways represented by Stat3 to support naive pluripotency and Erk to prepare cells for differentiation are possibly conserved. Therefore, we proposed that the developmental scheme in which the pluripotent epiblast cells go through a Stat3-dependent naive state to an Erk-dependent primed state before gastrulation might be conserved among amniotes (Fig. 4C). The shift in signal requirements for proliferation and survival suggest that molecular input into cell cycle machineries could also be changed during the transition. In contrast, transcription networks run by the factors downstream of signaling pathways could have been modified for the species-specific developmental events. In our model, amniotes generally establish the epiblast cell population based on the molecular interaction of Nanog inhibiting the hypoblast-driving gene circuit, while mammals added ICM/TE segregation governed by Pou5f1 to the default framework of development seen in birds (Fig. 5A). Further studies are awaited to supply the details of our models and to examine if they are applicable to non-amniotic vertebrates.

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