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REVIEW

Key features of the POU transcription factor Oct4 from an evolutionary perspective

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Abstract

Oct4, a class V POU-domain protein that is encoded by the *Pou5f1* gene, is thought to be a key transcription factor in the early development of mammals. This transcription factor plays indispensable roles in pluripotent stem cells as well as in the acquisition of pluripotency during somatic cell reprogramming. Oct4 has also been shown to play a role as a pioneer transcription factor during zygotic genome activation (ZGA) from zebrafish to human. However, during the past decade, several studies have brought these conclusions into question. It was clearly shown that the first steps in mouse development are not affected by the loss of Oct4. Subsequently, the role of Oct4 as a genome activator was brought into doubt. It was also found that the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) could proceed without Oct4. In this review, we summarize recent findings, reassess the role of Oct4 in reprogramming and ZGA, and point to structural features that may underlie this role. We speculate that pluripotent stem cells resemble neural stem cells more closely than previously thought. Oct4 orthologs within the POUV class hold key roles in genome activation during early development of species with late ZGA. However, in Placentalia, eutherian-specific proteins such as Dux overtake Oct4 in ZGA and endow them with the formation of an evolutionary new tissue—the placenta.

Keywords Oct4 · Zygotic genome activation · Reprogramming · Pluripotency · iPSCs · ESCs · NSCs

Introduction

The transcription factor Oct4 (Oct3, Oct3/4, Pou5f1), encoded by the *Pou5f1* gene, has held the attention of molecular and cellular biologists since its discovery in mammals more than 30 years ago due to its important roles in early embryogenesis and pluripotency [1, 2]. Oct4 is a member of the POUV class of proteins, which are involved in the regulation of early development. Proteins of this family contain a single POU domain that has flexible abilities in DNA recognition and mediates sequence-specific binding of DNA, as well as homo- and heterodimerization with other POU-family members [3–6]. This domain also facilitates the heterodimerization of Oct4 with its partner Sox2 [7]. The importance of Oct4 for pluripotent stem cells was confirmed by loss-of-function studies. Oct4-knockout mouse embryos exhibit arrested development before implantation at the blastocyst stage [8, 9]. The expression level of this transcription factor is under tight control, as either up- or downregulation of Oct4 expression leads to the differentiation of embryonic stem cells (ESCs) [10, 11].

Oct4 deserves special attention due to its critical role in the derivation of induced pluripotent stem cells (iPSCs) [12]. Several studies have attempted to replace Oct4 in the "Yamanaka's cocktail" of the transcription factors Oct4, Klf4, Sox2, and c-Myc (OKSM). While Klf4, Sox2, and c-Myc could be substituted by their homologs, Oct4 was postulated to be indispensable for reprogramming. However, recent works have shown that when a polycistronic vector and lentiviral system were used for iPSC derivation, reprogramming could be achieved with KSM and even with KS alone, although endogenous Oct4 expression was still crucial for enabling the cells to reach the pluripotent state [13, 14].

Debates are ongoing regarding the capability of Oct4 to bind closed chromatin and thereby participate in ZGA. In the past few years, Oct4 has been shown to bind nucleosomal DNA [15]; however, its role in ZGA in mammals remains

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unclear given that Oct4-deficient mice undergo normal early development [9, 16, 17].

To resolve these inconsistencies, we sought to take a deeper look into the functional features of the POUV class in general and Oct4 in particular. The newly emerged data on the potential of KSM reprogramming and the presence of these factors in neural stem cells (NSCs) could indicate that these cells resemble pluripotent cells more closely than previously thought. Furthermore, by comparing the role of Oct4 orthologs in ZGA in zebrafish and xenopus, we conclude that while these proteins do act as genome activators in species with late ZGA, proteins such as Dux induce ZGA much earlier in Placentalia, and thus overtake Oct4.

Oct4 structural features

The POU domain

Oct4 belongs to the family of transcription factors possessing the evolutionary conserved POU domain. The name POU originates from the first letters of the names of the founding members of the family—mammalian Pit1, Oct1, Oct2, and *C. elegans* Unc86 [3]. The POU domain itself consists of a POU-specific (POUs) subdomain and a POU-homeo (POUh) subdomain connected by a flexible linker that facilitates versatility in DNA recognition. The total length of the POU domain within the family is slightly variable, mostly due to linkers of different lengths; on average, the POU domain consists of 160 amino acids [18]. Within a typical POU-domain binding motif, the octamer sequence (ATGCAAAT), POUs recognizes the ATGC consensus sequence and POUh recognizes the AT motif. The presence of POUs is a distinctive feature of POU-family proteins, whereas POUh is a classic homeodomain that is ubiquitously present in multicellular organisms within proteins such as Hox [18]. Both POUs and POUh use the helix-turn-helix structure for DNA recognition, with helix3 positioned in the major DNA groove [19]. In the case of monomeric binding, POUs and POUh bind opposite sides of the DNA (Fig. 1a). As most of the crystallographic studies have been done with the Oct1 POU domain, we will refer to the residues of the corresponding Oct4 protein and its homologs with numbering starting from the beginning of the POU domain. Amino acids Q44, T45, and R49 within the POUs subdomain and amino acids R95, R97, V139, C142, N143, and Q146 within the POUh subdomain (Fig. 2a) are involved in the formation of DNA contacts [6, 7, 19]. These amino acids are highly conserved across all POU-domain proteins (Fig. 2a), suggesting functional conservation in DNA recognition. It is important to note that while POUs and POUh are involved mostly in interactions with major grooves, R95 and R97 at the beginning of POUh form contacts with one minor groove of the DNA and lie within the RKR(95)KR(97) motif, which in turn presents a nuclear localization signal [6, 20, 21].

The linker region of Oct4 is also important for the function of Oct4 in the self-renewal of ESCs and in pluripotency induction, although the data on its role in these processes varies. The Oct4 linker region has been demonstrated to harbor an additional alpha-helix and to contribute to protein–protein interactions [22]. It also contains negatively charged residues E78, E82, and E87, which presumably

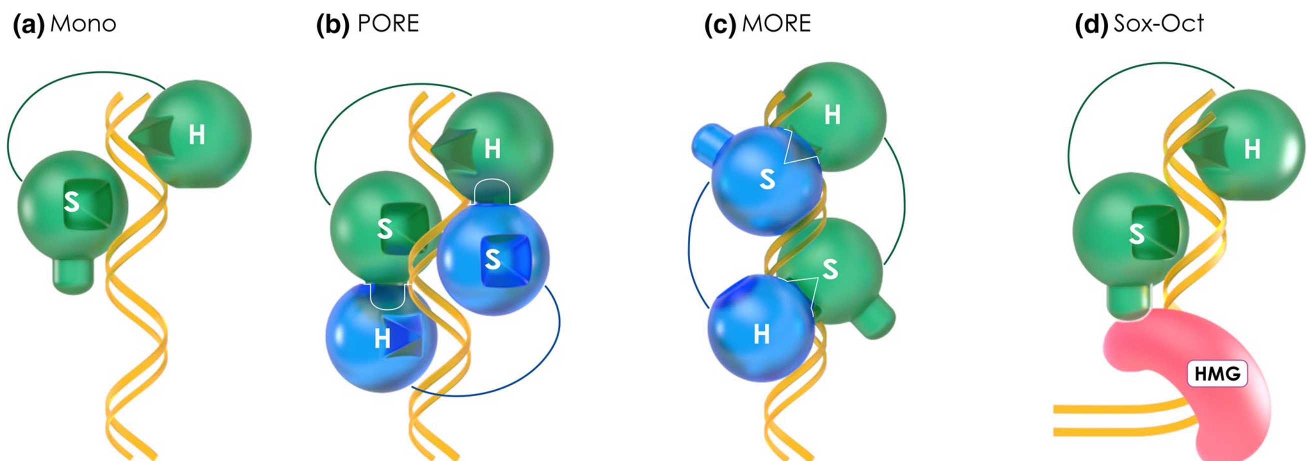

Fig. 1 POU-domain binding to DNA and dimerization. “S” stands for the POUs subdomain and “H” for the POUh subdomain. Pins and pyramids with corresponding recesses represent special contacts responsible for PORE and MORE binding, respectively. **a** Monomer POU DNA binding with opposite localization of subdomains, **b** POU homodimerization on the PORE, mediated by I21 of POUs; subdomains bind opposite sides of the DNA as in monomer binding, **c** POU heterodimerization on the MORE, mediated by S151; subdomains bind perpendicular faces of the DNA instead of opposite sides, **d** POU heterodimerization with the HMG domain on the Sox-Oct element, partially mediated by I21 from the POUs subdomain

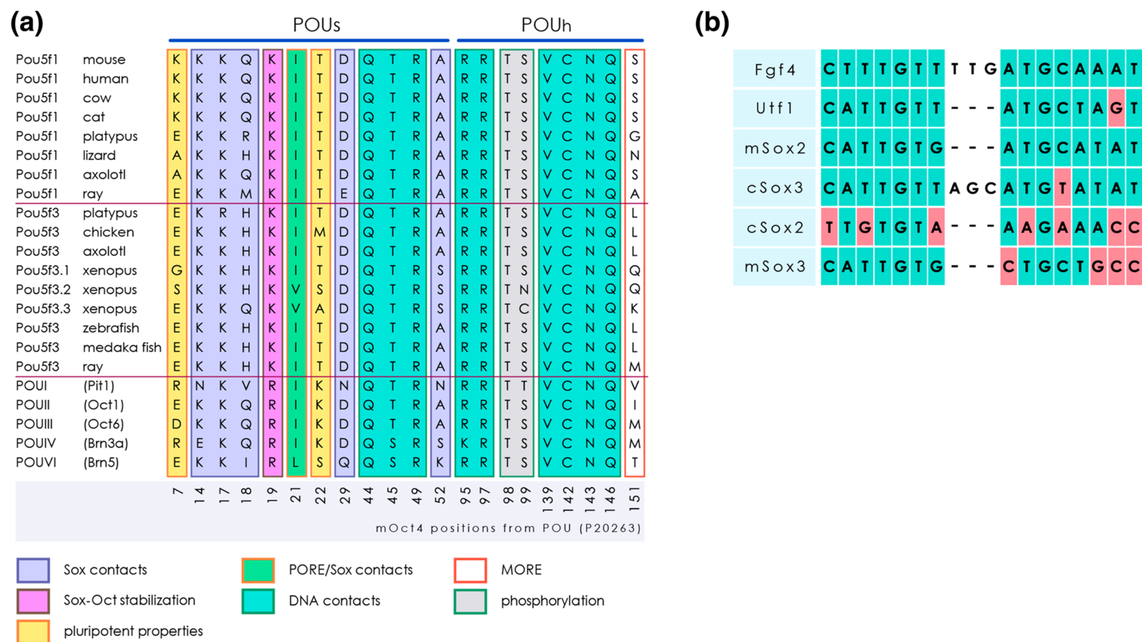

Fig. 2 **a** Conservation of functional amino acids in POUV proteins across vertebrates; three main subgroups of POU domain–containing proteins are represented (Pou5f1 orthologs and Pou5f3 orthologs from different species, and several examples of POUV paralogs); other classes of POU domain–containing proteins from mouse, **b** potential Sox-Oct element near chicken *Sox3* gene (mSox: mouse *Sox* gene, cSox: chicken *Sox* gene); *Fgf4* and *Utf1* regulatory regions were taken as examples; green-colored letters mean permissive nucleotides for SoxB or POUV binding, red-colored letters mean less suitable for HMG-POU binding

fine-tune Oct4-DNA contacts by shielding the positively charged R95 and R97 [21]. The substitution of E78, E82, and E87 by alanine has led to enhanced protein-DNA binding and improved reprogramming efficiency. On the other hand, recent work in which 5-AA depletion has removed E82 and E87 has also shown enhanced DNA binding but an inability of such modified Oct4 to induce and maintain pluripotency [23]. Finally, the replacement of the Oct4 linker to that of Oct6, which does not harbor an alpha-helix and E residues, could still induce pluripotency, albeit with a reduced efficiency—at 30% [24].

POU-POU dimerization

A hallmark of POU-containing proteins is their ability to form homodimers and heterodimers on specific DNA sequences. Due to the presence of a flexible linker, POU domains could form different structures during the process of dimerization, depending on the DNA sequence context [25]. There are two major DNA interfaces for POU homodimerization—the PORE (the Palindromic Oct factor Recognition Element—motif ATTTGAAATGCAAAT) [4] and the MORE (the *More* PORE—motif ATGCATATGCAT) [5] (Fig. 1b,c). In the PORE configuration, binding of POU domains to DNA is reminiscent of a monomer structure on the DNA surface—POUs and POUh within each protein are located oppositionally to each other (Fig. 1b) [6]. Dimerization of POU domains, in this case, is achieved mostly via the interaction of the POUs I21 (conserved in Oct4) from the first POU domain with the hydrophobic surface patch of POUh from the second POU domain. In the MORE configuration, POUs and POUh instead of binding on opposite sides of the DNA, bind at perpendicular faces of the DNA (Fig. 1c) [6, 25]. In this case, I160 of Oct1 plays a crucial role in interface formation via the interaction of this amino acid of POUh from the first POU domain with a hydrophobic cavity formed by POUs of the second POU molecule (Fig. 1c). Consequently, the substitution of I21Y disrupts dimerization on the PORE configuration but does not affect dimerization on the MORE configuration, whereas substitution of I160D leads to the opposite effect. Notably, the corresponding residue of Oct4 (S151) is also suitable for dimerization on the MORE configuration [5, 6], and this type of homodimerization is more common for Oct4 in vivo than on the PORE, as suggested by ChIP-seq analyses [26, 27].

Sox-Oct heterodimerization

Oct4, like other POU-domain proteins, can form homodimers on the PORE and MORE, but its preferred mode of DNA binding in vivo involves heterodimerization with Sox2. This type of dimerization is achieved through an interaction between the POU and HMG domains of Oct4 and Sox2,

respectively, on the so-called Sox-Oct motifs (CATTGT AATGCAAAT) (Fig. 1d) [24]. This interaction is facilitated mostly by I21 and D29 of the Oct4 POUs—two amino acids that are conserved in general within the POU family (Fig. 2a) [7]. K14, K17, Q18, and A52 from the POUs were shown to contribute to this contact [28]. In the Oct4-Sox2 complex, the POU domain adopts a configuration resembling that on the octamer site and PORE. Importantly, Sox2-Oct4 heterodimerization is crucial for pluripotency maintenance, and this complex is thought to regulate expression of genes such as *Fgf4*, *Utf1*, and *Nanog*, as well as their own genes—*Pou5f1* and *Sox2* [7, 29–33]. The cooperativity between Sox2 and Oct4 is also important in the context of pluripotency acquisition, as disruption of the Sox2-Oct4 dimerization interface abolishes iPSC formation [26]. There is also specific flexibility in Sox-Oct motif recognition, with the most common examples being the *Fgf4* and *Utf1* enhancers—in *Fgf4*, there is a 3-bp spacer between the Sox and Oct binding sites (CTTTGTTTGGATGCTAAT), whereas in the Utf1 regulatory element, the sites are closely juxtaposed (CATTGTTATGCTAGT) [30, 31]. Interestingly, different amino acids within Sox2 are engaged in heterodimerization with Oct4 on the *Fgf4* and *Utf1* enhancers [34].

The causes of equilibrium shift from homodimerization of Oct4 to heterodimerization with Sox2 on Sox-Oct motifs have been studied in several works. Jerabek et al. pointed out that while members of the majority of POU classes exhibit aliphatic residues M, V, or I at the very end of the POUh, Oct4 has a polar serine (S151) at this position, which is less suitable for homodimerization on the MORE [24]. Indeed, in titration experiments, the authors showed that Oct4 prefers the Sox-Oct formation because the S151 is not optimal for MORE-type dimerization, and that S151M substitution greatly improves Oct4 dimerization on the MORE. Consistent with this, the M151S substitution in Oct6 (POUIII class) decreased Oct6 homodimerization on the MORE to the Oct4 level. In vivo support for these results was obtained via ChIP-seq experiments during the course of iPSC derivation. Another study has shown the equilibrium shift from Sox-Oct motif to MORE binding of S151M Oct4 and the loss of M151S Oct6 homodimerization on the MORE interface, though mutant Oct6 did not demonstrate an increase in Sox-Oct motif occupation [26]. These results have pointed to an "evolutionary broken" POUh in Oct4—an idea that is supported by the observation that POUh is not involved in nucleosome distortion during the binding of Sox2-Oct4 to closed chromatin [15]. This statement is also in accordance with the evidence that POUIII-class proteins (Brn2, Brn4, and Oct6) could substitute for Oct4 in the reprogramming of mouse embryonic fibroblasts (MEFs) with the help of only a polycistronic vector that provides production of a fused POUIII-Klf4 protein with a subsequently inactive POUIII C-terminus [35]. However, this hypothesis is not supported by two more recent studies [23, 27]. The first study showed that substitution of the Oct4 POUh domain with that of Brn2 (POUIII-class) did not reduce the number of iPSC colonies but rather increased the number by fivefold [27]. Overall, this work demonstrated that an artificially "enhanced POU factor" (ePOU)—which featured Oct4 with T22R substitution, E78P in the linker region, and a Brn2-derived POUh—resulted in a 15-fold increase in the number of iPSCs during the reprogramming of MEFs, compared with wild-type Oct4. The second study also showed the possibility of MEF reprogramming by Oct4 with POUh domain from Brn2 (POUIII-class) and Brn3 (POUIV-class), albeit with reduced efficiency [23]. Regarding the S151, one may also refer to Oct4 orthologs of the POUV class. The farthest Oct4 ortholog of this class is Pou5f3 from zebrafish. ChIP-seq analysis of the Pou5f3 on the embryos of *Danio rerio* also showed the preferred dimerization of Pou5f3 with SoxB, as this analysis identified Sox-Oct, not the MORE, as the major DNA-binding motif [36]. It is notable that Pou5f3 has L in position 151 of the POU domain (Fig. 2a), which is aliphatic as in mammalian non-POUV class proteins. In sum, there is no conservation of residue 151 across POUV-containing animals, as this residue can be presented by both polar and aliphatic amino acids (Fig. 2a, Table 1). Moreover, this residue does not appear to predict preferential binding to Sox-Oct versus MORE in vivo.

Table 1 Sequences used in this work

Name	Species	NCBI/Uniprot ID
Pou5f1 mouse	*Mus musculus*	P20263
Pou5f1 human	*Homo sapiens*	Q01860
Pou5f1 cow	*Bos taurus*	O97552
Pou5f1 cat	*Felis catus*	NP_001166912
Pou5f1 platypus	*Ornithorhynchus anatinus*	NP_001229656
Pou5f1 lizard	*Anolis carolinensis*	XP_008120169
Pou5f1 axolotl	*Ambystoma mexicanum*	AAT09163
Pou5f1 ray	*Amblyraja radiata*	XP_032903387
Pou5f3 platypus	*Ornithorhynchus anatinus*	XP_028910026
Pou5f3 chicken	*Gallus gallus*	ABK27428
Pou5f3 axolotl	*Ambystoma mexicanum*	AGN30963
Pou5f3.1 xenopus	*Xenopus laevis*	NP_001081342
Pou5f3.2 xenopus	*Xenopus laevis*	NP_001079832
Pou5f3.3 xenopus	*Xenopus laevis*	NP_001081583
Pou5f3 zebrafish	*Danio rerio*	NP_571187
Pou5f3 medaka fish	*Oryzias latipes*	NP_001098339
Pou5f3 ray	*Amblyraja radiata*	NP_001371109
POUI (Pit1)	*Mus musculus*	Q00286
POUII (Oct1)	*Mus musculus*	P25425
POUIII (Oct6)	*Mus musculus*	P21952
POUIV (Brn3a)	*Mus musculus*	P17208
POUVI (Brn5)	*Mus musculus*	Q07916

Evidence points to a leading role of Sox2 in Oct4 recruitment to Sox-Oct motifs [37, 38]. POU proteins of classes I, II, and III also form dimers with SoxB-class proteins in vitro [24], consistent with the conservation of key amino acids that are responsible for these contacts (Fig. 2a). Overall, POUV class proteins exhibit a unique ability to heterodimerize with SoxB proteins in vivo, necessitating a closer look at the POUs subdomain and those amino acids contained therein that might shift the preference of POUV to an interaction with SoxB. The aforementioned study with fused POUIII-Klf4 proteins also indicated that the POUs plays a crucial role in reprogramming, as the substitution of the POUs of Oct4 with that of Brn4 led to a reduction of the number of iPSCs by about 30-fold [35]. The presence of K7 and, particularly, T22—which were not conserved in non-POUV-class proteins—in the Oct4 POUs was shown to be essential for ESC self-renewal. Substitution of these residues, along with the transfer of the linker sequence from Oct4, renders Oct6 capable of rescuing pluripotency in Oct4-deficient mouse ESCs [39]. There is currently no data on the functions of these residues, so additional studies are needed to clarify this issue. As for other Oct4 POUs-specific amino acids, K19 (K156 in the original paper) was proposed to be important for Oct4 stabilization within the Sox-Oct heterodimer [40]. This residue lies exactly in the interface that is responsible for Sox-Oct complex formation, and the K19N mutation impairs Sox2-Oct4 dimerization in vitro. A distinctive feature of POUV-class proteins is the presence of the K19 amino acid, which is strongly conserved from sturgeon to human (Fig. 2a). However, replacement of Oct4 K19 to a non-POUV residue (R) did not have any effect on Sox-Oct dimerization [40]. On the other hand, as we pointed out earlier, non-POUV proteins also showed an ability to form a Sox-Oct complex in vitro, so there is no discrepancy. It is also possible that K19 serves as a site for post-translational modifications in vivo, possibly reinforcing POUV dimerization with SoxB.

In mammals, there is also a well-known interaction of Oct4 with the SoxF-class member Sox17, driving the differentiation of ESCs into primitive endoderm [41]. The assembly of Oct4 and Sox17 occurs on the so-called "compressed" Sox-Oct motif (CATTGTATGCAAAT), which is 1-bp shorter than the canonical one (CATTGTcATGCAA AT). The compressed motif sterically prevents Sox2-Oct4 formation but allows Sox17-Oct4 assembly [42]. Interestingly, E122K substitution in Sox17 enables the protein to replace Sox2 in MEF reprogramming experiments [42], while E46L and K57E substitutions in Sox2 allow for Sox2-Oct4 assembly on the compressed motif [43].

Oct4 orthologs

Although POUV is an evolutionarily recent class, it exhibits differential functional conservation. In rescue experiments with mouse ESCs, human Oct4 was shown to be fully capable of substituting for the mouse ortholog [44] (Table 1). Within mammalian taxa, platypus Oct4 (Pou5f1) can rescue mouse ESCs, whereas opossum pou2 (Pou5f3) cannot. An avian homolog of Oct4, namely chicken POUV (cPouV), was shown to demonstrate approximately 50% of rescue capacity [45]. Interestingly, across amphibians, *A. mexicanum* (AmOct4) and *X. laevis* POUV-class members (Xlpou25/Pou5f3.2, Xlpou60/Pou5f3.3, Xlpou91/Pou5f3.1) demonstrated decent rescue capacity, at nearly 40%, on average, and Xlpou91 (Pou5f3.1), at nearly 100% [45, 46]. The linker region of Pou5f3.1 from Xenopus exhibits sequence similarity with mouse and human Oct4 and may contribute to Oct4's rescue ability [22]. Across Oct4-studied orthologs, the evolutionarily rather distant zebrafish and medaka Pou5f3 factors could not substitute for mouse Oct4 in ESC self-renewal [22, 44, 46]. Interestingly, the opposite is not true, as mouse Oct4 mRNA injected into maternally and zygotically Pou5f3-deficient embryos (MZspg) rescues zebrafish early development [47]. Similar results in iPSC generation were obtained with Oct4 orthologs—human and mouse Oct4 showed identical efficiency, Xlpou91 (Pou5f3.1) from xenopus was slightly less efficient—whereas AmOct4 (Pou5f1) and Ampou2 (Pou5f3) from axolotl, as well as Pou5f3 from zebrafish and medaka, generated hardly any iPSCs [48].

SoxB-protein conservation

SoxB-class proteins have a conserved protein interface for interaction with Oct4. Across mouse paralogs, Sox1, Sox3, and Sox15 can successfully rescue Sox2-deficient ESC self-renewal [49]. Astonishingly, even the SoxB member of *Drosophila melanogaster* SoxNeuro could replace Sox2, and, moreover, the rescued ESCs could contribute to mouse development [49]. This finding points to a crucial role of the regulatory elements of *SoxB* genes driving *SoxB* gene expression at specific times during development, rather than highlighting their structural differences. This statement is corroborated by evidence that in other vertebrates, different SoxB proteins such as Sox19b in zebrafish and Sox3 in Xenopus function like Sox2 in early mammalian development [50–53]. While the function of Oct4 and Nanog in bird pluripotency is unquestionable, there is little or no information on which SoxB protein performs a similar function in birds [45]. No autoregulatory Sox-Oct elements have been found near the *Sox2* gene in the chick genome (Fig. 2b) [54]. However, *Sox3* gene has one such element (CATTGTTAGC ATGTATAT) positioned 1,891 bp upstream of the TSS and

reminiscent of the mammalian *Fgf4* regulatory element with a 3-bp spacer in between the Sox and Oct motifs (Fig. 2b). Accordingly, Sox3, but not Sox2, is expressed in chicken oocytes and early developing embryos and probably cooperates with cPouV and Nanog in DNA-binding[55]. Collectively, these findings suggest that animals that harbor POUV-class proteins exhibit a novel regulatory network provided by POUV and perhaps by Nanog—but not by SoxB proteins—that operates in early development.

Oct4 modifications

Post-translational modifications (PTMs), studied mainly in mammals, have been found to modulate Oct4 function [40, 56–64]. Most are contained within non-conserved N- and C-termini outside of the POU domain and have been shown to mediate several Oct4 characteristics. Ubiquitination of K63 (the numbering in this section as in full-length mouse Oct4 protein) leads to Oct4 degradation and is, therefore, involved in Oct4 protein level control [63], whereas S111 phosphorylation increases Oct4 ubiquitination [61]. The stability of the Oct4 protein is mediated by K118 sumoylation [62, 65] and by S347 phosphorylation [59]. Residues T228 and S229 (T98 and S99 of the POU domain) deserve special attention due to their location within the conservative POUh subdomain (Fig. 2a) [56]. Their phosphorylation negatively influences Oct4 DNA-binding characteristics and decreases reprogramming efficiency. However, single T228 phosphorylation was shown to improve Oct4 stability in embryonic carcinoma cells (ECCs) and to increase ECC tumorigenicity [60]. As for S229, Saxe et al. showed that phosphorylation at this position affects the ability of Oct4 to form homodimers on the MORE and the PORE [57]. Oct4 modifications could also influence partner choice during heterodimerization. Phosphorylation at T343 leads to a stable Oct4 interaction with Sox2 and improved pluripotency maintenance while dephosphorylated Oct4 binds mostly to Sox17. This, in turn, leads to a high rate of spontaneous differentiation of ESCs into primitive endoderm and to the formation of teratomas of significantly reduced size; nevertheless, these teratomas contain derivatives of all germ layers [58].

Oct4 role in genome activation

The early development of multicellular organisms is accompanied by and is dependent upon widespread genome activation, called zygotic genome activation (ZGA). ZGA takes the baton from maternally provided transcripts to prepare the cells for subsequent differentiation into embryonic and extraembryonic tissues [66, 67]. ZGA is accompanied by the opening of thousands of loci, global genome acetylation, and demethylation (in mouse and human). Induction of differentiated cells into their early embryonic pluripotent state during iPSC generation is reminiscent of ZGA in terms of chromatin opening by the so-called “pioneer” factors [68]. Pioneer factors are thought to be the proteins that engage and thereby open nucleosome-occupied genomic DNA to attract other transcription and chromatin remodelling factors. POUV members are thought to display pioneer activity during ZGA in zebrafish, xenopus, and human, as well as in pluripotency induction. In the ensuing part of the review, we will discuss these features of POUV proteins and address issues regarding their ability in DNA opening.

POUV binding of closed chromatin

Several studies point to the ability of POUV proteins to bind nucleosome-occupied DNA, providing accessibility to other factors. Crystallographic analysis implies that POU proteins bind to the full octamer in the context of free DNA [7]. However, in the context of nucleosomes, Oct4 appears to bind to the so-called “partial motifs” [69]. Recent work has confirmed these results by structural analysis of the Sox2-Oct4 heterodimer binding to nucleosome DNA at Sox-Oct motifs [15]. This study showed that Sox2 and Oct4 bind to the entry-exit sites of nucleosome DNA and though Oct4 alone could release the DNA from the core histones, the presence of Sox2 synergizes the effect. Also, only the POUs domain was shown to be involved in the process of recognizing a partial ATGC motif, consistent with the results of Soufi et al. Binding to entry-exit nucleosome sites may suggest the occupation of free DNA during the process, which is called “nucleosome breathing” and refers to when the nucleosome is not located in a fixed position but slides within a certain area [70]. However, the results of the structural analysis indicate that there is still a closed Oct4 motif that is bound cooperatively by Oct4 and Sox2 [15]. Thus, several factors permitting closed DNA binding at the Sox-Oct element by Oct4, nucleosome breathing, and Oct4 cooperativity with Sox2 all contribute to this process (Fig. 3a). Though the crucial role of the cooperation between Sox2 and Oct4 in nucleosome DNA binding was shown, Oct4 alone also plays an important role in this process at sites besides the Sox-Oct motif. A very recent article by Roberts et al. points that in the case of Oct4 binding to nucleosome DNA, the relative POUs and POUh orientation is critical [23]. The authors showed that several depletions at the beginning of POUh result in shortening of the distance between the POUs and POUh, cancelling of the binding to nucleosome DNA but not affecting binding to naked DNA. This modification also abolished Oct4 function in pluripotency induction and maintenance. Additionally, depletions within the linker region in turn led to enhanced nucleosome and naked DNA binding but also to abolishment of pluripotency maintenance and induction [23]. POUV binding to

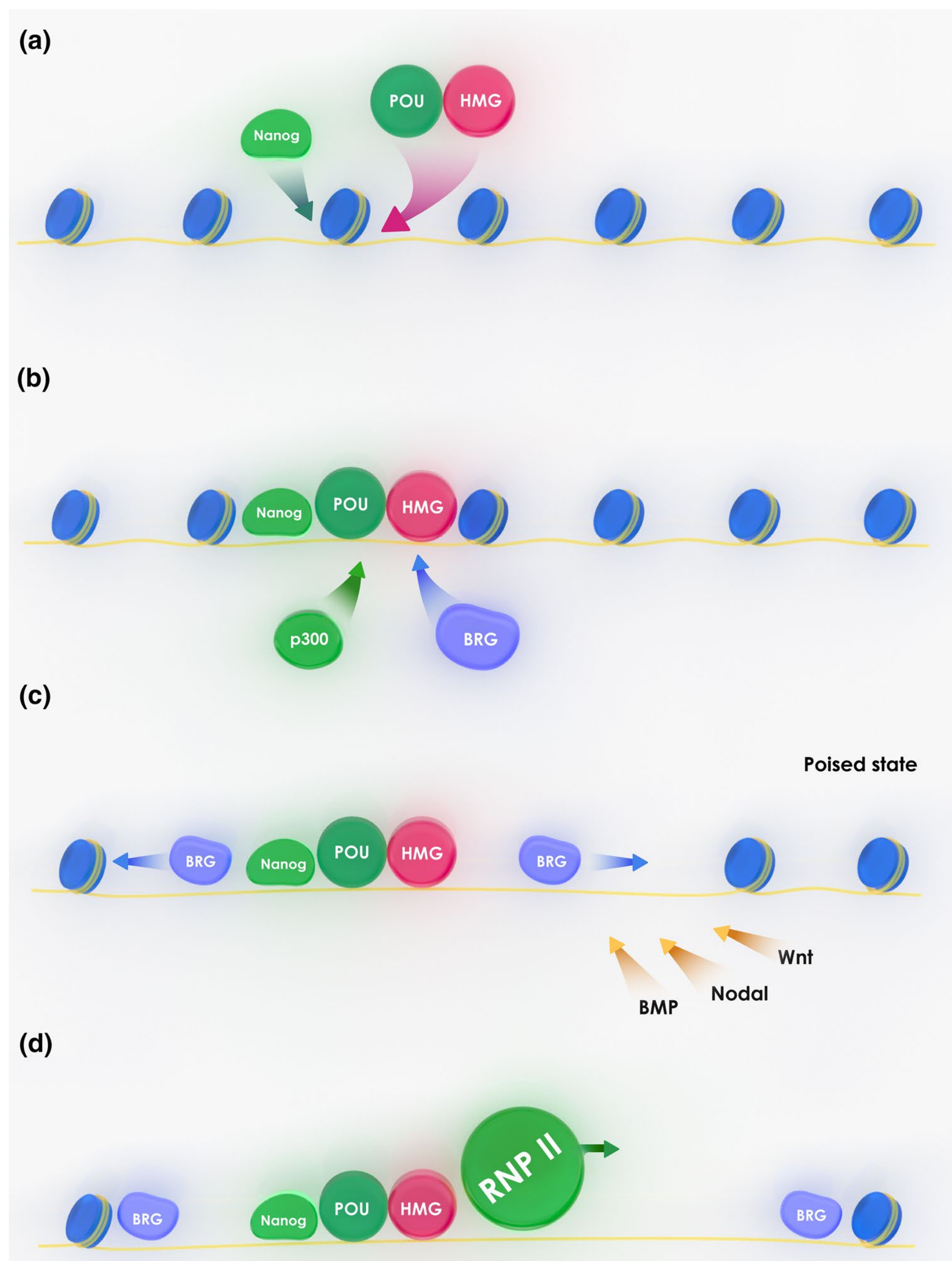

Fig. 3 POUV role in genome activation: **a** Initial local DNA opening by HMG-POU complex, along with Nanog, **b** recruitment of chromatin modifiers such as BRG and acetyltransferases to this locus, **c** expansion of DNA opening with the subsequent emergence of a poised state, which is characterized by an ability to respond to different external signals, **d** recruitment of transcriptional machinery and the onset of transcription

closed DNA fits well with the idea of specific "high nucleosome affinity regions" (HNAR) based on Pou5f3, Nanog, and Sox19b genome binding during zebrafish ZGA [71]. It has been demonstrated that loss of Pou5f3 and Sox19b leads to failure to activate 75% of zygotic genes and that Pou5f3 overexpression in MZspg mutants (Pou5f3 knockouts) rescues the expression of most ZGA genes [36, 72]. The HNAR hypothesis is based on the mechanism whereby transcription factors cooperate indirectly (without direct protein–protein interactions) to compete with nucleosomes for DNA binding [73]. Veil et al. suggested that HNARs are not characterized by a special arrangements of DNA motifs, but as a 600-bp sequence that is structurally attractive for both nucleosomes and transcription factors [71]. This attractiveness is achieved via predicted nucleosome occupancy and specific values of propeller twist and GC content. Using a zebrafish model, the authors found that before ZGA, Pou5f3 and Nanog non-specifically (regardless of motif enrichment) reduce nucleosome occupancy and bind specific motifs after ZGA [71]. Meers et al. recently provided support for the nonspecific action of pioneer factors [74]. Those authors showed that during endoderm specification, FoxA2 establishes a more universal motif when it binds nucleosome DNA with some variations, compared to a precise motif when it binds to free

DNA. ChIP-seq data have shown that Oct4 binds numerous genome locations that do not contain cognate Octamer, MORE, or Sox-Oct motifs. Such genome sites can comprise up to half of all sites during reprogramming [26].

While these studies indicate that POUV proteins play a role in providing accessible chromatin to other factors, several studies downplay the role of these proteins. During the reprogramming of mouse fibroblasts, initial binding of Oct4 has been shown to occur mostly in somatically active yet accessible enhancers to displace somatic transcription factors from these loci [75]. Both Pou5f3 in zebrafish and Oct4 in mouse were shown to be dependent upon BRG1 (Smarca4a in zebrafish) for making chromatin accessible [76, 77] (Fig. 3b). BRG1 is a protein that can remove nucleosomes from DNA. In these works, POUV factors were shown to be engaged mostly at distal regions while other factors engaged promoters. Zebrafish Pou5f3 prefers sperm-inherited methylated regions, which lose their accessibility upon Pou5f3 knockdown. However, they also lose this feature upon Smarca4a knockdown [76]. In mouse ESCs, of the Oct4 binding sites, which were shown to rely on Oct4 for accessibility, 76% also rely on BRG1 for accessibility. Interestingly, after Oct4 depletion, Sox2 and Nanog were unable to bind to their common sites [77]. Additional preliminary work on zebrafish revealed that Nanog, Pou5f3, and Sox19b (NPS) are also involved in the establishment of H3K27ac and H3K18ac modifications by acetyl-transferase recruitment (Fig. 3b). By analyzing distinct loci, the authors demonstrated that artificial recruitment of p300 to regulatory regions of *asb11* and *her5* could bypass the NPS requirement in the activation of these genes during ZGA (Liyun Miao, …, Antonio J. Giraldez, 2020, bioRxiv). This work, like the previous one, also points to a crucial role of NPS for distal regions rather than for promoters—half of the distal enhancers and only 5% of the promoters lost their accessibility upon NPS knockdown. Of note, the dependence of p300 on Oct4, Sox2, and Nanog for binding to DNA was also shown in mouse ESCs [78]. Reprogramming studies of mouse B-cells demonstrated that the addition of the transcription factor CEBPA, which is thought to make chromatin transiently more accessible, leads to roughly 100-fold increase in OKSM-dependent reprogramming, resulting in 95% of B-cells being successfully converted into iPSCs [79, 80]. In sum, it appears that an evolutionarily conservative feature of POUV factors is to engage nucleosome-occupied DNA and release nucleosomes at distinct regions. By doing this, POUV members could indeed be named pioneer factors; however, additional transcription factors and chromatin remodellers are needed for completion of loci opening, preparing the loci for further activation (discussed below).

Zygotic genome activation

Studies on zebrafish, xenopus, mouse, and human show that the provisioning of DNA accessibility during early embryogenesis does not necessarily assume transcriptional activation, but rather prepares chromatin to receive external developmental cues [50, 81–83] (Fig. 3c). During zebrafish development, chromatin accessibility has been shown to be independent of RNA-polymerase II but dependent upon Pou5f3, Nanog, and Sox19b binding, predicting future transcription (Fig. 3d) [81]. A similar study in the early development of xenopus revealed that Pou5f3 (Pou5f3.2 and Pou5f3.3) and Sox3 prepare chromatin for upcoming Wnt, Nodal, and BMP signals. The authors showed that among 708 genes that show response to these signals, 268 are dependent upon Pou5f3 and Sox3 [50]. Wu et al. pointed to the presence of an unusual pre-ZGA open chromatin state without detectable transcription in both mouse and human. These regions were poised for further developmental activation [82]. The provisioning of accessible DNA by Oct4 for external signals was also noted in both the maintenance and differentiation of mouse ESCs. STAT3 and Smad1 co-occupy multiple sites with Oct4, and following Oct4 knockdown, their binding to these sites is diminished [78]. At the same time, a set of Oct4-occupied/DNAse-low accessible genes related to Wnt and retinoic acid (RA) signalling were identified, implying their readiness for corresponding differentiation signals. For example, Oct4-occupied enhancers of Wnt-dependent T/Bra and RA-dependent Hoxa1 also showed absolute Oct4-dependent activity [84].

Debates surround the role of Oct4 during mammalian ZGA. Oct4 is thought to contribute to ZGA in human but not in mouse [67]; however, this difference does not appear to be conclusive. While ZGA is observed at the 2-cell stage in mouse [85] and the 8-cell stage in human [86], the onset of Oct4 expression occurs at a common time point—before the 8-cell stage—in mouse [87], bovine [88], and human [89, 90]. Studying DNase I hypersensitive site (DHS) dynamics revealed opening of 7471, 15,914, and 57,450 of those sites during the 2-, 4-, and 8-cell stages in mouse, respectively, while 729, 4582, and 39,813 DHSs became open at the same stages in human [91]. During this process in human, 25% of DHSs were dependent on Oct4. At the same time, a leading role of the Nfya transcription factor during mouse ZGA was noted, as Nfya knockdown resulted in 28% of DHS loss at the 2-cell stage [83]. Of note, in mouse, Oct4 still contributes to DHS onset (27% of all DHS), but at the 8-cell stage, like in human [83]. Interestingly, there is a significant number of Nfya binding sites at 2-cell–stage DHSs in mouse, whereas about the same number is reached only by the 8-cell stage of human development [91]. This finding may suggest that Nfya overtake Oct4 in mouse embryogenesis and hence, turns on ZGA earlier, i.e., at the 2-cell stage. Finally,

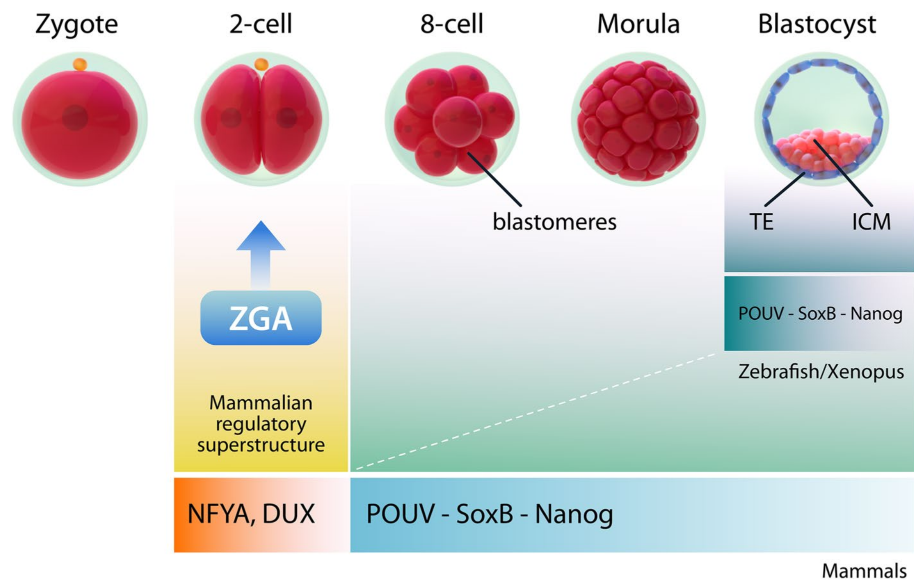

Fig. 4 Mammals are characterized by the presence of a new regulatory superstructure that provides earlier ZGA for novel tissue (trophectoderm) differentiation. Activators such as Nfya and Dux overtake Oct4-Sox2-Nanog in mammalian ZGA and hence allow for an early launch of the first differentiation event, in this case trophectoderm specification. At the blastocyst stage, the inner cell mass is reminiscent of a time point of zebrafish and xenopus ZGA

regarding early genome activation, the contribution of eutherian-specific Dux proteins is more pronounced than that of Oct4 in both mouse and human [92–94]. Dux protein is detected at the 2-cell stage in mouse and at the 4-cell stage of human embryogenesis. It activates cleavage-specific genes such as Zscan4, MERVL, miR-344, and Snai1 and participates in chromatin remodelling. Relatively recently, specific 2-cell–like ESCs were established [95]. Among their notable features were expression pattern, MERVL transcription, ability to contribute to both extraembryonic and embryonic tissues that were like blastomeres of 2-cell–stage embryos, and, surprisingly, absence of Oct4, Nanog, and Sox2 expression. Furthermore, Dppa2 and Dppa4 were shown to regulate this state by directly activating *Dux*, thereby launching the 2C-like status [96]. Considering the ability of these ESCs to contribute to both embryo and extraembryonic cell types, they can be considered as totipotent, and thus, Dux can be called the master genome activator. Interestingly, non-mammalian animals are characterized by far more cleavage divisions before ZGA—14 cycles in drosophila, 10 cycles in zebrafish, and 13 cycles in xenopus versus 1 and 3 cycles in mouse and human, respectively [67]. POUV factors in zebrafish and xenopus undoubtedly make the major contribution to ZGA [36, 50, 72]. It is known that mouse and human are characterized by the presence of placenta—novel extraembryonic tissue whose evolutionary advent coincides with the transition to early ZGA. Mouse development until the blastocyst stage is not compromised by Oct4 knockout, i.e., ZGA is launched and trophectoderm is established [9, 16, 17]. Mutant embryos demonstrate initial expression of the pluripotency marker Nanog but fail to establish primitive endoderm. Only further development of Oct4-deficient mouse is associated with pluripotency shutdown, upregulation of trophectoderm markers, and presence of metabolic disorders [97]. Upon Oct4 knockout in human, a significant portion of embryos still develops to the blastocyst stage; however, these human embryos do not show Nanog expression, unlike Oct4-knockout mouse embryos [98]. Thus, Oct4 protein becomes indispensable for embryonic development at stages that could be compared with those of zebrafish and xenopus, i.e., 1 k-cell stage and mid-blastula transition, respectively. In this way, perhaps during the evolutionary emergence of trophectoderm, earlier ZGA was needed for this new type of differentiation, and instead of POUV, SoxB, and Nanog, earlier genome activators, such as Dux and Nfya, took over this function. We thus propose a model in which Oct4 continues to be a global genome activator and gatekeeper of this active genome state in mammals, but only after ZGA. This switch of the mode of action of Oct4 might be evolutionarily coupled to the emergence of the novel tissue—the placenta (Fig. 4).

Pluripotency induction

The discovery of induced pluripotent stem cells (iPSCs) demonstrated the possibility of reverting adult somatic cell fates into one of an early pluripotent state with the help of defined factors—Oct4, Klf4, Sox2, and c-Myc (OKSM) [12, 99]. Although further studies demonstrated the ability to substitute Klf4, Sox2, and c-Myc by their homologous proteins, Oct4 was still the only factor that could not be replaced by its POU-containing homologs (Brn2, Brn4, Oct6, etc.). Several works, in which Oct4 was omitted from the reprogramming mixture, were nevertheless aimed at the direct activation of endogenous Oct4 [100–102]. The application of a common monocistronic retroviral system for reprogramming with the help of the POU-domain proteins Brn2, Brn4, and Oct6 from the POUIII family in place of Oct4 resulted in

trans-differentiation of MEFs into induced neural stem cells (iNSCs) [35, 103], which was expected due to the involvement of these proteins in neuroectoderm development and not in pluripotency. However, human fibroblasts were reprogrammed by OCT6-KSM, though iPSC formation was delayed when compared with OCT4-KSM [104]. Intriguingly, the mutations I21Y/D29R in OCT6, which have been shown to eliminate the interaction of Oct4 with Sox2 [7, 26], abolished any iPSC formation by OCT6-KSM, pointing to the ability of OCT6 to interact with SOX2 in vivo, which to date has been observed only for OCT4. On the other hand, ChIP-seq analysis revealed the MORE as a preferred binding motif type for OCT6, so additional data is needed to clarify this issue. A subsequent article by these authors also showed that inhibition of H3K79 methyltransferase, the TGF-beta pathway, and lysine-specific histone demethylase during reprogramming results in an epigenetically more permissive state [105]. This state facilitates the reprogramming of human fibroblasts with the help of not only almost any POU-containing factor (along with KSM), but also PRDM14, OTX2, SIX3, NANOG, GATA3, and several other factors in place of Oct4 [105]. As it was mentioned above, during MEF reprogramming by POUIII-class proteins, successful iPSC reprogramming could be achieved only with polycistronic vectors that expressed fused POUIII-Klf4 protein with subsequently inactive POUIII C-terminus, which is incompatible with POU factor dimerization on the MORE [35]. These vectors are based on human immunodeficiency virus (HIV) (referred hereafter to simply as lentiviruses). Further important works showed that this system also allowed MEF reprogramming by only KSM and even KS, i.e., without Oct4 [13, 14]. An et al. have pointed out that a polycistronic system facilitates the stoichiometry and cooperativity of Klf4 and Sox2, as well as supports the reprogramming of cells regardless of germ layer origin [13]. In the second study, the leading role in KSM-mediated reprogramming was assigned to lentiviruses, as separately transduced lentiviral KSM also produced iPSCs, albeit at a lower efficiency [14]. The authors pointed out that the coexpression of Sox2 and c-Myc rapidly leads to silencing of commonly used for reprogramming moloney murine leukemia virus (MMLV)-based retroviral vectors (referred hereafter to simply as retroviruses). Retrovirus usage precludes KSM reprogramming, whereas lentivirus usage somehow allows for bypassing this effect. The authors also point out that a high proliferation rate is favourable for pluripotency induction, as c-Myc and GATA factors, as well as fibroblast immortalization by SV40 Large T antigen, similarly facilitate KS-mediated reprogramming. In this regard, the participation of OCT6 and several other factors in reprogramming of human cells with retroviruses appears to reflect overcoming of retrovirus silencing and enhancement of proliferation rather than direct activation of the pluripotency program [104, 105]. No data currently exist on KSM-mediated reprogramming of human fibroblasts with the lentiviral polycistronic system. As lentiviruses are sometimes considered less suitable for transduction of human cells, finding suitable conditions for such reprogramming may be a challenge. Of note, there are some culture conditions that provide a "naïve" status for human ESCs and iPSCs [106, 107]. Considering that Klf4 is a common marker for this type of pluripotency, deploying these culture conditions may also facilitate KSM-mediated reprogramming of human cells. In mouse, a greater developmental potential was shown by the tetraploid (4 N) complementation assay for iPSCs derived with KSM compared with those obtained with OKSM. The authors further showed that this difference is likely due to massive off-target gene activation by Oct4 and imprinting abnormalities. An interesting observation of this work was the indirect reprogramming route in the middle of reprogramming promoted by OKSM, with an upregulation of neuroectoderm markers, whereas KSM promoted a more straightforward route to pluripotency. In this context, several facts point to a close relationship between pluripotent and neural stem cells. The initial presence of Oct4 alongside KSM reprogramming has been shown to prompt fibroblasts toward the iNSCs state [108]. A distinctive feature of NSCs is the expression of the three common reprogramming factors—Klf4, Sox2, and c-Myc [37], and thus, the addition of Oct4 is sufficient to prompt them toward the iPSC state [109]. Considering that KSM can reprogram fibroblasts into iPSCs, the real developmental potential of NSCs must be thoughtfully reassessed. iPSC-derived NSCs have been shown to spontaneously reactivate the pluripotent state in vitro [110]. Moreover, approximately 20 years ago, a series of publications pointed to NSCs as the cell type that can contribute to not only the neural lineage, but also all germ layers [111–114]. Thus, there could be two explanations: either NSCs represent a specific type of pluripotent cell or they could transform themselves into *bona fide* Oct4-positive pluripotent cells under certain conditions. The idea is indirectly supported by a recent in vivo work, which shows reprogramming of neuroectodermal precursors during neural crest establishment in mouse embryogenesis [115]. Oct4 is reactivated in premigratory cranial neural crest cells (CNCCs), which in turn give rise to not only neuroectoderm derivatives but also mesenchymal lines, e.g., bone, cartilage, and smooth muscle. Of note, Oct4 re-expression was observed in smooth muscle cells within atherosclerotic lesions and found to be important for the atheroprotective functions of these cells [116]. In sum, the above examples suggest that Oct4-dependent reprogramming occurs not only artificially in vitro, but also naturally during development and, under certain pathological conditions, in adult organisms. NSCs appear to possess the lowest epigenetic barrier for that, and it would be of high interest to determine

whether Oct4-mediated reprogramming occurs also in the developing and in the adult neural system.

Conclusions

Although some non-class V POU proteins demonstrate the ability to form dimers with SoxB proteins in vitro and thus have all the necessary amino acids for this contact, only members of the POUV class clearly prefer to cooperate with SoxB proteins in living cells. This shift in DNA binding from the MORE-type POU homodimerization appears to be mediated by some structural features in the POUs subdomain. The amino acids K7, K19, and T22, which are conserved across the POUV class, deserve special attention in this respect, as they may provide clues to these features. Recent studies have shown that reprogramming with the help of HIV-derived lentiviral vectors somehow bypasses the retroviral silencing program, which is true for MMLV-based retroviral vectors, and facilitates pluripotency induction even without Oct4 in Yamanaka's cocktail. During the reprogramming of human cells with the help of retroviral vectors, OCT4 was shown to be substituted by OCT6, and upon further addition of some inhibitors even by transcription factors of different families. Oct4, despite being redundant during reprogramming as an exogenous factor, remains essential for pluripotency acquisition as an endogenous factor. There is accumulating evidence that Oct4 plays a role during in vivo reprogramming and in this regard, NSCs appear to be the most likely candidate whose developmental potential should be re-evaluated from this point of view. Debates surround the participation of Oct4 in ZGA and the opening of closed chromatin. Studies of the past decade have pointed out that, indeed, POUV proteins can bind nucleosome-occupied DNA in cooperation with SoxB members and recruit chromatin remodellers and acetyltransferases. We hypothesize here, that in mammals, although Oct4 is not a major player in initial ZGA, it does contribute to chromatin activation after trophectoderm specification. We also conclude that in Placentalia, factors such as Dux and Nfya overtake Oct4, Sox2, and Nanog in launching the major ZGA wave, thereby preparing the genome for trophoblast specification.

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