Received: 3 January 2022 | Revised: 25 February 2022 | Accepted: 14 March 2022

DOI: 10.1002/bdr2.2007

REVIEW ARTICLE

Birth Defects Research | Society for Birth Defects Research & Prevention | WILEY

Regulation of *Oct4* in stem cells and neural crest cells

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Funding information
Andrew McDonough B+ Foundation (Ronald J. Parchem); CPRIT Scholar in Cancer Research RR150106 (Ronald J. Parchem); National Institutes of Health R01-HD099252; R01-HD098131 (Ronald J. Parchem); V Scholar in Cancer Research #V2017-017 (Ronald J. Parchem)

Abstract

During embryonic development, cells gradually restrict their developmental potential as they exit pluripotency and differentiate into various cell types. The POU transcription factor Oct4 (encoded by *Pou5f1*) lies at the center of the pluripotency machinery that regulates stemness and differentiation in stem cells, and is required for reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). Several studies have revealed that *Oct4* and other stemness genes are also expressed in multipotent cell populations such as neural crest cells (NCCs), and are required to expand the NCC developmental potential. Transcriptional regulation of *Oct4* has been studied extensively in stem cells during early embryonic development and reprogramming, but not in NCCs. Here, we review how *Oct4* is regulated in pluripotent stem cells, and address some of the gaps in knowledge about regulation of the pluripotency network in NCCs.

KEYWORDS

craniofacial defects, neural crest cells, Oct4, pluripotency, reprogramming, stem cells

1 | INTRODUCTION

Neural crest cells (NCCs) are a unique, ectoderm-derived, multipotent cell population that give rise to both ectodermal derivatives such as neurons and glia and ectomesenchymal derivatives such as chondrocytes and osteocytes (reviewed in Le Douarin, Creuzet, Couly, & Dupin 2004; Cebra-Thomas et al., 2013). Understanding the molecular mechanisms that govern NCC multipotency may unlock the etiologies of many developmental anomalies that arise during neural crest development, including craniofacial defects, cardiac defects, and pediatric cancers, such as neuroblastoma (T. S. Sato et al., 2019). We are just beginning to understand some of the key molecular factors that regulate mammalian NCC development. Several studies have suggested that NCCs express the pluripotency machinery to expand their developmental potential and give rise to ectomesenchymal derivatives such as chondrocytes and osteocytes (Scerbo & Monsoro-Burq, 2020; Zalc et al., 2021). Thus, the gene regulatory networks of pluripotent embryonic stem cells (ESCs) likely have essential roles in NCCs.

Pluripotent cells of the mammalian epiblast express the core transcription factors, Oct4, Sox2, and Nanog,

Abbreviations: cNCCs, cranial neural crest cells; EpiSCs, epiblast stem cells; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; lncRNAs, long noncoding RNAs; MEFs, mouse embryonic fibroblasts; mESCs, mouse embryonic stem cells; miRNAs, microRNAs; NCCs, neural crest cells; NPB, neural plate border; NRs, nuclear receptors; NT, neural tube; OSKM, Oct4, Sox2, Klf4, and c-Myc.

which define and maintain the pluripotent state (Osorno & Chambers, 2011). During differentiation, expression of *Oct4* and other pluripotency genes is lost as lineage-specific gene programs are activated. Exit from pluripotency and lineage segregation is carefully coordinated by several signaling cues and transcriptional factors. Differentiation into different lineages was once considered a unidirectional process; pluripotent stem cells at the peak of Waddington's landscape traveled downhill toward their ultimate cell fate, progressively restricting their developmental potential along the way (Waddington, 1957). This paradigm was challenged in 1962 when Sir John Gurdon generated a fully functional tadpole by transplanting the nucleus of a differentiated intestinal epithelium cell from an adult frog into an enucleated unfertilized egg (Gurdon, 1962). This landmark result suggested that a fully differentiated cell retains the genetic memory and potential to give a complete organism when placed in an appropriate environment. Thirty years later, somatic cell nuclear transfer technology (SCNT) was applied to mammals to clone Dolly the sheep (Wilmut, Schnieke, McWhir, Kind, & Campbell, 2007).

This work laid the groundwork for Yamanaka and Takahashi, who discovered that the introduction of four transcription factors, Oct4, Sox2, Klf-4, and c-Myc (OSKM), could convert a somatic cell into an induced pluripotent stem cell (iPSC; Takahashi & Yamanaka, 2006). Several groups have since identified different combinations of pluripotency factors sufficient to reprogram somatic cells (reviewed in Takahashi & Yamanaka, 2016). These studies fundamentally changed our concept of cellular plasticity by showing that differentiated cells could dedifferentiate in the presence of critical components of the pluripotency program. Later work showed that differentiated cells can also transdifferentiate—transform directly into another differentiated cell type without first passing through a pluripotent state (reviewed in Grath & Dai, 2019). Collectively, these groundbreaking findings created new avenues in regenerative biology and revealed insights into mechanisms of dedifferentiation that can occur in normal development and disease. Here, we review different mechanisms and factors that regulate the pluripotency network in stem cells and development, with a focus on *Oct4*.

2 | MAIN

2.1 | Regulation of the Oct4 pluripotency network in ESCs

Transcription factors regulate cell fate decisions by acting as molecular switches to activate or silence gene expression programs during development. A study in mouse embryonic stem cells (mESCs) showed that a two-fold increase in *Oct4* expression pushed mESCs to differentiate into primitive endoderm and mesoderm, whereas loss of *Oct4* expression caused differentiation into trophectoderm (Niwa, Miyazaki, & Smith, 2000), suggesting that the levels of pluripotency transcription factors must be tightly maintained for stem cell self-renewal. Reprogramming of somatic cells into iPSCs requires ectopic expression of pluripotent factors both in vivo and in vitro. Important to clinical translation, the delivery of pluripotency factors for in vivo reprogramming needs to be tightly regulated as it can lead to tumor formation (Simpson, Olova, & Chandra, 2021). Thus, it is not surprising that Oct4 levels are regulated by several mechanisms to maintain pluripotency in ESCs (Table 1).

2.1.1 | The DNA methylation status of *Oct4* regulatory elements

Direct reprogramming to a pluripotent state requires genome-wide changes in chromatin composition, such as global DNA demethylation (Maherali et al., 2007; Mikkelsen et al., 2008). Integrative genomic analysis during reprogramming revealed that DNA hypermethylation of pluripotency-related genes resulted in inefficient reprogramming in partially reprogrammed cell lines (Mikkelsen et al., 2008). Indeed, inhibition of DNA methylation using 5-aza-cytidine in the partially reprogrammed cell lines resulted in a rapid and stable transition to a complete pluripotent state (Mikkelsen et al., 2008). *Oct4* transcription is regulated by at least three well-studied sites; the proximal promoter, the proximal enhancer, and the distal enhancer (Niwa, 2007; Yeom et al., 1996). Consistent with an important role in maintaining pluripotency, the DNA of these regulatory elements was found unmethylated in ESCs and methylated in somatic cells (Hattori et al., 2004). During somatic cell reprogramming, the methylation status of the *Oct4* locus directly correlated with reprogramming efficiency and selection of iPSCs (Mikkelsen et al., 2008).

DNA methyltransferases (Dnmt) are a family of enzymes that catalyze the transfer of methyl groups to DNA. Dnmt3a and Dnmt3b were sufficient and required to methylate the proximal enhancer and promoter elements of *Oct4* (Figure 1) and *Nanog* during the differentiation of mESCs (J. Y. Li et al., 2007). Moreover, complete loss of *Dnmt3a* and *Dnmt3b* in mouse embryos resulted in abnormal expression of *Oct4* and *Nanog* at E9.5 (both genes are silenced by E9.5 in wild-type embryos; J. Y. Li et al., 2007). In contrast to Dnmt enzymes, the ten-eleven translocation (Tet) proteins, TET1 and TET2, played an

TABLE 1 Summary of pluripotency genes regulated by different factors and mechanisms in various systems

Stem genes	Regulators	Class	Mechanism	Model	Reference
Oct4 and Nanog	Dnmt3a and Dnmt3b	DNA methyltransferases	DNA methylation of proximal enhancer and promoter	mESCs/mouse embryos	J. Y. Li et al. (2007)
Oct4, Nanog	Tet1	DNA demethylases	Facilitates DNA demethylation of Oct4 and prevents hypermethylation of Nanog promoter	mESCs	Gao et al. (2013); Ito et al. (2010)
Oct4	G9a	Histone methyltransferase	Promotes methylation of H3K9 residues at the Oct4 promoter and recruits Dnmt3a for further DNA methylation	Mouse embryos/mouse neural stem cells	Feldman et al. (2006); Ma et al. (2008)
Oct4	Jhdm2a	Histone demethylase	Global DNA demethylation resulted in Oct4 reactivation	Mouse embryos/mouse neural stem cells	Ma et al. (2008)
Nanog, Oct4, Tbx3, Esrrb Bmp4, Tcl1, Klf4, and Klf5	Paf1c-Set1 complex	Histone methyltransferase	Maintains active H3K4me3 at the promoters which results in further recruitment of histone acetyltransferases for active H3K27ac	mESCs	Ding et al. (2009)
Oct4	SF-1/NR5A1 and LRH-1/NR5A2	Orphan nuclear receptors	Maintains Oct4 expression possibly by recruiting activating chromatin remodelers and transcription factors	Human embryonal carcinoma NCCIT cells, embryonic carcinoma P19 cells, and mESCs	Gu, Goodwin, et al. (2005); Yang et al. (2007)
Oct4, Nanog and Sox2	GCNF/NR6A1	Orphan nuclear receptors	Negatively regulates Oct4 expression by recruiting Dnmt enzymes and promoting DNA methylation of proximal enhancer and promoter	mESCs and mouse embryo	Fuhrmann et al. (2001); Gu et al. (2006); Gu, Goodwin, et al. (2005); Gu, LeMenuet, et al. (2005); N. Sato et al. (2006); Yang et al. (2007)
Oct4	COUP-TFII/ NR2F2	Orphan nuclear receptors	Negatively regulates Oct4 expression by binding to the proximal promoter	mESCs and mouse embryo	Rosa and Brivanlou (2011)
Oct4, Sox2, and Nanog	Sox2-Oct4 complex	Pluripotency factors	Binds to cis-regulatory enhancer elements to activate transcription	mESCs, hESCs, mouse embryos	Chew et al. (2005); Kuroda et al. (2005); Okumura-Nakanishi et al. (2005); Tomioka et al. (2002)

(Continues)

TABLE 1 (Continued)

Stem genes	Regulators	Class	Mechanism	Model	Reference
Oct4	Nanog	Pluripotency factors	—	Mouse neural stem cells	Ma et al. (2008)
Oct4	Sall4	Pluripotency factors	Binds to distal enhancer element and activates Oct4 transcription	mESCs	Zhang et al. (2006)
Oct4, Sox2, and Nanog	Tcf3	Transcription factor (terminal component of Wnt signaling pathway)	Binds to promoters and acts as transcriptional repressor	mESCs	Cole et al. (2008)
Oct4	miR-302	MiRNA	Positively regulates Oct4 by targeting cell cycle regulator AKT1	hESCs	H. L. Li et al. (2016)
Oct4, Sox2 and Klf4	miR-145	MiRNA	Degrades/destabilizes mRNA transcripts	hESCs	Xu et al. (2009)
Oct4, Sox2, and Nanog	linc-ROR, and Oct4-pseudogene 5	Long intergenic noncoding RNAs/ pseudogene	Acts as miRNA sponge and prevents miR-145 mediated targeting	hESCs/endometrial carcinoma	Bai et al. (2015); Wang et al. (2013)
Oct4 and Nanog	AK028326 (Oct4-activated) and AK141205 (Nanog-repressed)	LncRNAs	Forms a feedback regulatory loop with Oct4 and Nanog	mESCs	Sheik Mohamed et al. (2010)
Oct4	as-Oct4-pg5	LncRNAs	Recruits histone methyltransferase Ezh2 to Oct4 locus, resulting in increased repressive H3K27me3 levels suppressing Oct4 transcription	MCF-7 breast cancer cell line (human)	P. G. Hawkins and Morris (2010)

essential role in initiating DNA demethylation in mESCs (Gao et al., 2013; Ito et al., 2010; Koh et al., 2011; Tahiliani et al., 2009). *Tet1* and *Tet2* were highly expressed in mESCs consistent with a globally demethylated state in naïve pluripotency (Ito et al., 2010; Koh et al., 2011). Loss of *Tet1* disrupted ES cell self-renewal and differentiation into different lineages consistent with the role of Oct4 in maintaining pluripotency and lineage specification (Ito et al., 2010; Koh et al., 2011; Takahashi & Yamanaka, 2016). Loss of *Tet1* also resulted in decreased RNA and protein levels of Nanog, Oct4, and Sox2 in ESCs (Ito et al., 2010). Tet1 replaced Oct4 in OSKM-mediated reprogramming of mouse embryonic fibroblasts (MEFs), while MEFs treated only with the three SKM factors failed to reprogram (Gao et al., 2013). Indeed, the addition of Tet1 to OSKM-mediated reprogramming significantly increased *Oct4* reactivation by enhancing demethylation of the enhancer and promoter regions (Gao et al., 2013). Together, these results suggest that the DNA methylation status of the *Oct4* promoter must be carefully regulated by the opposing roles of Dnmt and Tet enzymes in ESCs and during reprogramming.

2.1.2 | Dynamic changes in histone methylation of the *Oct4* regulatory elements

A comprehensive analysis of transcriptional and epigenetic changes during reprogramming revealed that DNA

methylation changes at promoters of genes occurred late during reprogramming (Polo et al., 2012). Pluripotency genes such as *Oct4* and *Nanog* became demethylated at later stages as well (Polo et al., 2012). Chromatin analysis of active H3K4me3 (trimethylation of the 4th lysine residue of histone H3 protein) and repressive H3K27me3 (trimethylation of the 27th lysine residue of histone H3 protein) histone methylation marks revealed dynamic changes in histone methylation patterns at the promoters of genes consistent with changes in the transcriptome during early stages of reprogramming (Polo et al., 2012). *Oct4* and *Nanog*, which were bivalently (H3K4me3 and H3K27me3) marked in MEFs, acquired H3K4me3 marks accompanied by a loss of H3K27me3 marks before DNA demethylation occurred at the promoter (Polo et al., 2012). These results suggest that changes in histone methylation precede DNA demethylation and are crucial in determining the transcriptional activation status of the *Oct4* locus during reprogramming.

Upon exit from pluripotency, active H3K4me3 histone modifications were removed from the *Oct4* promoter (Topalovic, Schwirtlich, Stevanovic, & Mojsin, 2017), followed by the addition of repressive histone marks, such as H3K9me3 (trimethylation of the 9th lysine residue of histone H3 protein) and H3K27me3 (Feldman et al., 2006; K. Wang, Chen, Chang, Knott, & Cibelli, 2009), and further stabilized by DNA methylation to repress *Oct4* transcription (Mikkelsen et al., 2008). H3K9me3 remains a major barrier to cellular reprogramming. Chromatin marked by H3K9me3 remains inaccessible to most regulatory factors and transcriptional machinery, in contrast to other heterochromatin markers, such as H3K27me3, where DNA remains accessible to general transcription factors and a paused RNA polymerase (reviewed in Becker, Nicetto, & Zaret, 2016). Suppression of the CAF-1 complex (which maintains H3K9me3) in MEFs resulted in the activation of the endogenous *Oct4* promoter independent of OSKM-induced reprogramming (Cheloufi et al., 2015). These results suggest that removal of H3K9me3 marks from the *Oct4* promoter may be the first critical epigenetic change required during reprogramming, followed by removal of H3K27me3 and DNA demethylation.

Histone methyltransferase and demethylase enzymes are responsible for maintaining the histone methylation and demethylation status, respectively. The histone methyltransferase G9a induces methylation of H3K9 residues, resulting in heterochromatin formation and transcriptional repression (Tachibana, Sugimoto, Fukushima, & Shinkai, 2001; Tachibana et al., 2002). Knocking down G9a enhanced reprogramming of neural cells by speeding up *Oct4* reactivation (Ma, Chiang, Ponnusamy, Ming, & Song, 2008). In contrast, the overexpression of histone demethylase Jhdm2a, which promotes transcriptional activation by catalyzing demethylation of H3K9 residues (Yamane et al., 2006), increased *Oct4* reactivation during the reprogramming of neural cells (Ma et al., 2008). These results suggest that the coordinated activity of opposing histone-modifying enzymes regulates the reactivation of *Oct4* expression during reprogramming (Figure 1). Furthermore, G9a was required for H3K9 methylation followed by DNA methylation of the *Oct4* promoter (Feldman et al., 2006). In the absence of G9a, DNMT3A was not recruited to the *Oct4* locus (Feldman et al., 2006). These results further support the idea that histone methylation changes precede DNA methylation of the *Oct4* locus. It appears that the down-regulation of G9a during the early stages of reprogramming remains the key to *Oct4* reactivation and efficient reprogramming (Feldman et al., 2006). While extensive studies have been done to determine how G9a regulates different physiological processes and its downstream targets, little is known about the factors that regulate G9a expression.

After removal of repressive histone and DNA methylation marks during reprogramming, the *Oct4* proximal promoter must be modified with active histone marks to maintain *Oct4* expression in iPSCs (Polo et al., 2012). A genome-wide screen led to the identification of factors within the Paf1 complex that had the strongest effect on *Oct4* expression and played an important role in maintaining ESC identity (Ding et al., 2009). Paf1C was bound to promoters of many pluripotency genes, including *Oct4*, and served as a platform to attract other histone modifiers (Ding et al., 2009). Knocking down Paf1C decreased levels of H3K4me3 at pluripotency genes such as *Oct4* with no changes at lineage-specific genes (Ding et al., 2009). Simultaneous knockdown of Paf1C with Set1 complex (maintains H3K4me3) resulted in the loss of *Oct4* expression (Ding et al., 2009). These results suggest that Paf1C controls pluripotency by directly maintaining H3K4me3 levels at promoter regions of pluripotency genes, including *Oct4*, by synergistically working with the Set1 complex (Figure 1). H3K4me3 can also recruit downstream effectors, including chromatin remodelers which can lead to further gene activation and repression (reviewed in Berger, 2007). For instance, H3K4me3 recruits the histone acetyltransferase protein NuA3 (acetylates histone H3 protein) to increase chromatin accessibility (Martin, Grimes, Baetz, & Howe, 2006; Figure 1). Indeed, histone acetylation is generally associated with transcriptionally active genes (reviewed in Kuo & Allis, 1998). Genome-wide analysis of histone acetylation and methylation modifications revealed that the pluripotency factor genes such as *POU5F1 (OCT4)*, *SOX2*, and *NANOG* showed hyperacetylation of H3K27

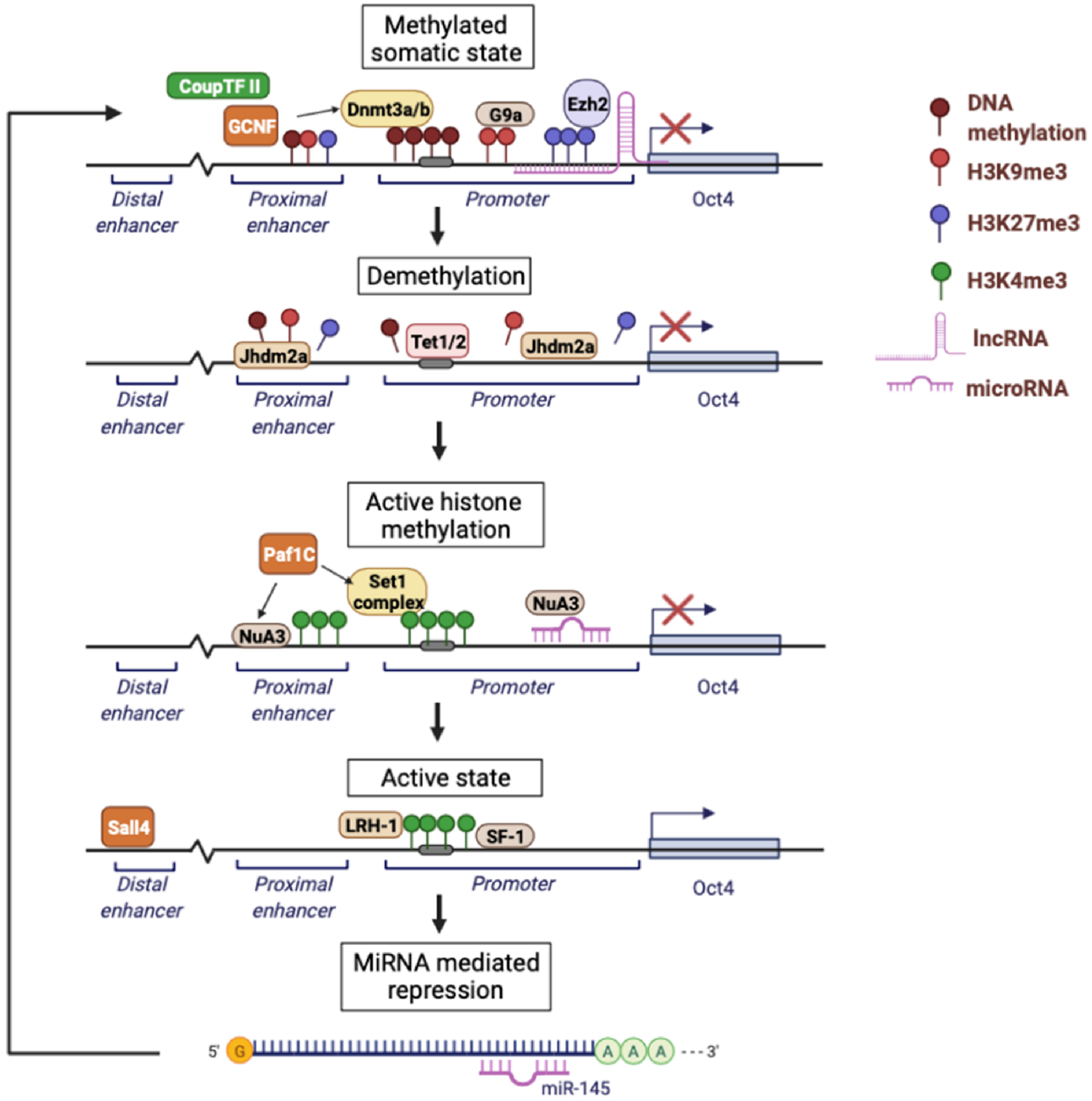

FIGURE 1 Regulation of *Oct4* is a sequential process governed by several genetic, epigenetic, and noncoding factors. *Oct4* locus was silenced in the somatic state by DNA and histone methylation (H3K9me3 and H3K27me3) marks maintained by epigenetic modifiers such as Dnmt3a (J. Y. Li et al., 2007), G9a (Feldman et al., 2006; Ma et al., 2008), and Ezh2 (P. G. Hawkins & Morris, 2010). These methylases were recruited to the *Oct4* locus by orphan NRs such as GCNF (Fuhrmann et al., 2001; Gu et al., 2006; Gu, Goodwin, et al., 2005; Gu, LeMenuet, et al., 2005; N. Sato et al., 2006; Yang et al., 2007) and COUP-TFII (Rosa & Brivanlou, 2011) and lncRNAs (P. G. Hawkins & Morris, 2010). Activation of the *Oct4* locus was regulated by demethylating enzymes such as Jhdm2a (Ma et al., 2008) and Tet proteins (Gao et al., 2013; Ito et al., 2010). Removal of inactive methylation marks increased accessibility of the *Oct4* locus for other regulatory factors such as Paf1C, Set1 complex, and NuA3 which increased active methylation marks (H3K4me3 and H3K27ac) at the *Oct4* regulatory elements (Ding et al., 2009). Addition of active methylation groups and recruitment of other transcriptional regulators such as Sall4 (Zhang et al., 2006), LRH-1, and SF-1 (Gu, Goodwin, et al., 2005; Yang et al., 2007) promoted active transcription of the *Oct4* locus. *Oct4* was posttranscriptionally regulated by miRNAs such as *miR-145*, resulting in the downregulation of *Oct4* levels during differentiation (Xu et al., 2009)

residues (acetylation of the 27th lysine residue of histone H3 protein or H3K27ac) in hESCs which was lost upon differentiation and replaced by H3K27me3 (R. D. Hawkins et al., 2011). Collectively, these studies suggest that Paf1C and H3K4me3 can play a crucial role in determining the status of *Oct4* transcription and maintaining the balance between stemness and differentiation in ESCs. Future studies investigating mechanisms regulating epigenetic modifiers in the pluripotent state may clarify the crosstalk controlling the dynamic chromatin accessibility at the *Oct4* locus.

2.1.3 | Coregulation of the *Oct4* locus by nuclear receptors

The cis-regulatory elements near *Oct4* contain binding sites for several orphan nuclear receptors (NRs), which maintain *Oct4* expression in ESCs or inhibit expression during differentiation and embryogenesis (Fuhrmann et al., 2001; Gu, Goodwin, et al., 2005; Yang et al., 2007). For example, the orphan NR steroidogenic Factor 1 (SF-1/NR5A1), activated *Oct4* transcription in pluripotent teratoma cells and was essential during late organogenesis (Yang et al., 2007). However, during early embryogenesis, another orphan NR, liver receptor homolog-1 (LRH-1/NR5A2), directly regulated *Oct4* expression (Gu, Goodwin, et al., 2005). *LRH-1* and *Oct4* were co-expressed in the epiblast and ESCs (Gu, Goodwin, et al., 2005). Loss of *LRH-1* resulted in the loss of *Oct4* expression and early embryonic death (Gu, Goodwin, et al., 2005; Figure 1). Further investigation is required to determine the precise mechanisms by which SF-1 and LRH-1 positively regulate *Oct4* in ESCs. In contrast, orphan NRs such as germ cell nuclear factor (GCNF/NR6A1), negatively regulated *Oct4* during differentiation (Fuhrmann et al., 2001; Gu, LeMenuet, et al., 2005). Retinoic acid-induced differentiation of *GCNF* knockout ESCs resulted in the loss of repression of *Oct4*, *Nanog*, and *Sox2* (Gu, LeMenuet, et al., 2005). GCNF binding silenced *Oct4* expression by recruiting DNA methylating enzymes such as DNMT3A/B to the proximal enhancer and promoter during differentiation (Gu, Le Menuet, Chung, & Cooney, 2006; N. Sato, Kondo, & Arai, 2006; Figure 1). In ESCs, OCT4 repressed expression of the orphan NR, *COUP-TFII/NR2F2*, which repressed *Oct4* transcription by binding to the proximal promoter during differentiation, forming a feedback loop (Mullen, Gu, & Cooney, 2007; Rosa & Brivanlou, 2011). Together, these studies highlight the critical role of orphan NRs in maintaining the pluripotent and differentiated states of ESCs by regulating *Oct4* transcription.

2.1.4 | Feedback regulation between signaling pathways and core pluripotency network

The transcription factors that comprise the core pluripotency network often form complexes with each other to coregulate transcription of their target genes as well as to autoregulate themselves and each other by feedback regulation. How these factors synergize to maintain the pluripotent state in ESCs remains understudied. Functional studies in mESCs have revealed that the SOX2-OCT4 complex occupies and coregulates *Oct4*, *Sox2*, and *Nanog* by forming an autoregulatory circuitry (Chew et al., 2005; Kuroda et al., 2005; Okumura-Nakanishi, Saito, Niwa, & Ishikawa, 2005; Tomioka et al., 2002). Overexpression of *Nanog* along with the histone demethylase Jhmd2a significantly improved endogenous *Oct4* reactivation and the reprogramming efficiency of NSCs (Ma et al., 2008). Indeed, *Oct4*, *Sox2*, and *Nanog* comprised the group of pluripotency genes that were expressed later during the second wave of transcriptional changes and conferred a stable pluripotency state during reprogramming (Polo et al., 2012). This category of genes included several other DNA-binding factors, such as *Sall4* (Polo et al., 2012). In an effort to identify a combination of transcription factors for somatic cell reprogramming, devoid of OSKM, a study identified a seven-factor (7F) system that could reprogram somatic cells into iPSCs with similar high quality and efficiency (B. Wang et al., 2019). This 7F system included the pluripotency gene *Sall4*, which, remarkably, was required for both opening and closing of chromatin during reprogramming (B. Wang et al., 2019). Functional assays indicated that SALL4 activated *Oct4* expression by binding to its distal enhancer element in ES cells (Zhang et al., 2006; Figure 1). These results suggest that the interconnected autoregulatory loop formed by pluripotency factors plays an important role in the maintenance of pluripotency and efficient somatic cell reprogramming.

The core pluripotency network is tightly regulated by extrinsic signaling pathways governing different pluripotency states. The pluripotent state of naïve mESCs is regulated by LIF, BMP, Wnt, and FGF signaling pathways, while that of primed EpiSCs is governed by TGFβ and FGF signaling pathways (reviewed in Mossahebi-Mohammadi, Quan, Zhang, & Li, 2020). These signaling pathways bring developmental cues directly to the core pluripotency circuitry to maintain the balance between stemness and differentiation in ESCs and during early mouse development (reviewed in Mossahebi-Mohammadi et al., 2020). For instance, T-cell factor-3 (Tcf3), a terminal component of the Wnt signaling pathway, maintained the balance between stemness and

differentiation in mESCs (Cole, Johnstone, Newman, Kagey, & Young, 2008). *Tcf3* knockout mESCs showed an increase in the expression of the core pluripotency genes—*Oct4*, *Nanog*, and *Sox2* (Cole et al., 2008), suggesting an important role of Tcf3 in coordinating exit from pluripotency. TCF3 was shown to occupy its promoter and that of the core pluripotency genes to form an autoregulatory feedback network (Cole et al., 2008). These studies highlight the fact that the pluripotency network is tightly regulated by a complex network involving multiple different signaling cues and transcription factors.

2.1.5 | Noncoding RNAs in Oct4 regulation

A significant portion of transcripts are not translated into proteins and are referred to as noncoding RNAs. Advances in RNA-sequencing techniques and functional assays have expanded our knowledge about different classes of noncoding RNAs and their critical function in development and disease. However, relatively little is known about noncoding RNAs that can activate *Oct4* transcription. MicroRNAs (miRNAs) post-transcriptionally silence gene expression by targeting mRNA transcripts, and they can also activate transcription by binding to enhancers and promoters (reviewed in Catalanotto, Cogoni, & Zardo, 2016). Screening for double-stranded RNAs (dsRNAs) that can bind to the *Oct4* promoter region led to the identification of a synthetic small activating RNA (saRNA), dsOCT4-622, that activated and increased *Oct4* transcription in human adipose-derived stem cells (J. Wang et al., 2015). While this study holds promise in developing small RNA-mediated reprogramming of somatic cells, it remains unknown if there are any endogenous small RNAs such as miRNAs that can activate *Oct4* transcription during development and reprogramming. MiRNAs can positively regulate *Oct4* expression indirectly by targeting other genes as well. For instance, *miR-302* promoted the self-renewal of human embryonic stem cells (hESCs) by targeting cell-cycle regulator *AKT1* and maintaining *OCT4* expression (H. L. Li et al., 2016). Pluripotency genes are also targets of miRNA-mediated silencing during differentiation. Luciferase reporter assays showed that *OCT4*, *SOX2*, and *KLF4* were directly targeted by miRNA *miR-145* in hESCs (Xu, Papagiannakopoulos, Pan, Thomson, & Kosik, 2009; Figure 1). In contrast, a long intergenic non-protein coding RNA (lincRNA), *linc-ROR*, and *Oct4-pseudogene 5* acted as miRNA sponges and prevented core pluripotency transcription factors such as *OCT4*, *NANOG*, and *SOX2* from *miR-145*-mediated suppression in hESCs (Bai et al., 2015; Y. Wang et al., 2013). Altogether, these results suggest a broader role for noncoding RNAs in regulating the expression of core pluripotency network genes.

Long noncoding RNAs (lncRNAs) are another class of noncoding RNAs that can modulate transcription programs by recruiting epigenetic modifiers at gene loci, serving as scaffolds for protein complex assembly and function, regulating miRNA activity, and affecting transcript stability and maturation (reviewed in Statello, Guo, Chen, & Huarte, 2021). Genome-wide transcriptome analysis in combination with chromatin occupancy analysis of pluripotency transcription factors in mESCs identified lncRNAs that were directly targeted by Oct4 and Nanog (Sheik Mohamed, Gaughwin, Lim, Robson, & Lipovich, 2010). In turn, these lncRNAs modulated the expression of *Oct4* and *Nanog* in a regulatory feedback loop (Sheik Mohamed et al., 2010). How these lncRNAs regulate pluripotency circuitry remains elusive. A lncRNA antisense to Oct4-pseudogene 5, *as-Oct4-pg5*, suppressed transcription of *Oct4* in MCF-7 breast cancer cell line (P. G. Hawkins & Morris, 2010). The lncRNA *as-Oct4-pg5* directed histone methyltransferase Ezh2 to the *Oct4* promoter resulting in increased H3K27me3 levels and heterochromatin formation at the *Oct4* locus (P. G. Hawkins & Morris, 2010). Further investigation is required to determine how *as-Oct4-pg5* recruits Ezh2 to the *Oct4* promoter, perhaps by RNA–protein interactions. In general, the role of lncRNAs in the context of reprogramming and Oct4 regulation requires further investigation.

2.2 | Neural crest cells challenge the accepted paradigm of cellular potential during development

Following specification at the neural plate border (NPB), NCCs undergo epithelial-to-mesenchymal transition (EMT), delaminate, and migrate throughout the embryo, giving rise to cells that contribute to several tissues in the body, such as the craniofacial skeleton, heart, enteric nervous system, melanocytes, and smooth muscle (Couly, Grapin-Botton, Coltey, Ruhin, & Le Douarin, 1998; Creazzo, Godt, Leatherbury, Conway, & Kirby, 1998; Le Douarin, 1982; Le Douarin & Smith, 1988; Le Douarin & Teillet, 1973; Le Lièvre & Le Douarin, 1975; Le Lievre, Schweizer, Ziller, & Le Douarin, 1980). Based on the origin along the anterior/posterior body axis, NCCs are divided into four subpopulations: cranial, vagal, trunk, and sacral NCCs. Cranial and trunk NCCs are the only subpopulations of the neural crest known so far that can give rise to ectomesenchymal cells, such as chondrocytes

and osteocytes (reviewed in Cebra-Thomas et al., 2013; Le Douarin et al., 2004).

NCCs are unique to vertebrates; however, the transcriptional circuitry that regulates NCC development is not constrained to vertebrates and is found in invertebrates as well. So, how did vertebrates evolve to expand the developmental potential of NCCs in order to form a highly advanced "head," endowing them with a greater advantage in the ecosystem compared to invertebrates? A recent study applied phylogenetic analysis and functional studies to reveal that evolution of the *VENTX/NANOG* gene family endowed NCCs with multipotency in vertebrates (Scerbo & Monsoro-Burq, 2020).

Prior studies had shown that pluripotency factors such as *pouV* (Oct4), *ventx* (Nanog), and *sox2* were expressed in the neural plate border region during development in *Xenopus* (frog) embryos (Morrison & Brickman, 2006; Rogers, Archer, Cunningham, Grammer, & Casey, 2008; Scerbo et al., 2012). Knocking down these pluripotency factors resulted in anterior neural defects and posterior truncations consistent with the contribution of NCCs to these tissues (Morrison & Brickman, 2006; Rogers et al., 2008; Scerbo et al., 2012). Further studies showed that pluripotency factors such as Oct4, Sox2, and Nanog were expressed in mouse NCCs and alluded to the role of pluripotency genes in the development and differentiation of NCCs (Hagiwara et al., 2014; Kikuchi et al., 2011). These studies used *P0-Cre* (Schwann cell-specific protein)/*Floxed-EGFP* mice to isolate EGFP+ cells from the iris stromal region (Kikuchi et al., 2011) or the craniofacial, and trunk region (Hagiwara et al., 2014) to perform sphere formation assay in order to assess their stem cell-like properties. Immunofluorescence staining (detects and visualizes protein expression) revealed expression of the pluripotency factors such as OCT4, NANOG, SOX2, and NESTIN in the spheres (Hagiwara et al., 2014; Kikuchi et al., 2011). Later, a study in mouse trunk NCCs showed that OCT4, NANOG, and SOX2 co-occupied regulatory elements of genes involved in the formation of trunk NCCs such as *Foxd3*, *Sox9*, and *Sox10* in a Wnt signaling-dependent manner (Fujita, Ogawa, & Ito, 2016). Knockdown of these pluripotency factors decreased expression levels of *FoxD3* and *Sox10* in trunk NCCs, suggesting a role for the pluripotency network in regulating NCC development (Fujita et al., 2016). Collectively, these studies highlighted the role of pluripotency factors in regulating NCC genes and maintenance of NCCs. However, it was still unclear whether the pluripotency factors were expressed in NCCs in vivo and during what stage of embryonic development.

The NCC gene regulatory network comprises several transcription factors, and it is well accepted that NCCs represent a highly heterogeneous cell population that expresses a combination of different transcription factors (Simões-Costa & Bronner, 2015). Techniques such as in situ hybridization and immunolabeling have allowed investigators to look at transcript and protein expression of pluripotency and NCC genes in intact embryonic tissue, but with relatively limited quantitative resolution. To visualize the transcriptional heterogeneity in NCCs, a recent study coupled multiplex single-molecule fluorescence in situ hybridization with machine learning to examine the expression of NCC and pluripotency genes at a single-cell resolution in vivo in chicken embryos (Lignell, Kerosuo, Streichan, Cai, & Bronner, 2017). The study revealed that not all cells with a neural crest gene signature expressed pluripotency genes (Lignell et al., 2017). Only one of the cell clusters in the dorsal neural tube with a premigratory NCC gene signature expressed pluripotency genes (Lignell et al., 2017). Interestingly, lateral to these cells there was another population of cells that expressed pluripotency genes but had a neural gene signature (Lignell et al., 2017). These results revealed that not all the cells in the dorsal neural folds that give rise to NCCs may express the pluripotency genes. The question remained whether the expression of pluripotency factors was required for the formation of both ectodermal and ectomesenchymal NCC derivatives?

The role of pluripotency factors in regulating NCC proliferation was further strengthened by a study in mouse cranial NCCs in association with folate metabolism (Mohanty et al., 2016). Folate deficiency results in neural tube defects (NTDs), such as spina bifida and anencephaly; hence, folate supplementation is highly recommended to women of reproductive age (Centers for Disease Control and Prevention, n.d.). One of the key questions in the field is to understand how folate metabolism regulates the development of neural tube and prevents NTDs. Functional studies showed that supplementation of folate rescued the proliferation and differentiation defect of NCCs in a mouse model of embryonic NTDs (Ichi et al., 2010, 2012; Nakazaki et al., 2008). FRα is a receptor for folic acid which upon binding of folic acid, translocates to the nucleus and acts as a transcription factor to regulate downstream genes (Mohanty et al., 2016). Treatment of a cranial NCC (cNCC) line, O9-1, with folic acid, increased expression of pluripotency factors, including *Oct4*, *Sox2*, *Klf4*, and *Trim71 (*Mohanty et al., 2016*)*. Chromatin-binding assays revealed that the FRα bound to regulatory elements of these pluripotency genes following FA treatment and promoted active transcription. This was further confirmed by enrichment of active H3K27ac modifications and p300 occupancy at enhancer elements of these pluripotency genes (Mohanty et al., 2016). FRα also downregulated miRNAs such as *miR-138* and *let-7* that targeted *Oct4* and *Trim71*, respectively (Mohanty

et al., 2016). In turn, knocking down *Oct4* and *Trim71* prevented folate mediated rescue of NCC proliferation defect in the NTD mouse model (Mohanty et al., 2016), which suggested that expression of pluripotency factors is required to maintain proliferation of NCCs during development.

Recent studies in *Xenopus* and mouse provided definitive evidence supporting the role of the pluripotency factors Oct4 and Nanog in NCC specification and ectomesenchyme development. Knocking down *ventx2* (Nanog) in *Xenopus* embryos disrupted early NCC specification and differentiation into ectomesenchyme without affecting NCC migration or differentiation into melanocyte and sensory lineages (Scerbo & Monsoro-Burq, 2020). The *ventx2* mutants displayed reduced craniofacial skeleton (Scerbo & Monsoro-Burq, 2020). The study further showed that *ventx2* could promote the expression of other pluripotency genes in early NCCs in cooperation with NCC/NB-specific transcription factors (Scerbo & Monsoro-Burq, 2020). Corroborating these results, ablation of *Oct4* function in cNCCs in mouse embryos caused craniofacial defects such as loss of frontonasal mass and absence of nasal processes (Zalc et al., 2021). Similar to the study in *Xenopus* (Scerbo & Monsoro-Burq, 2020), the ectodermal derivatives of cNCCs, such as neurons and glia, remained unaffected by loss of *Oct4* expression (Zalc et al., 2021). Together, these results demonstrated the role of the pluripotency network in endowing NCCs with ectomesenchymal potential in vertebrates across species.

Traditional views of embryonic development follow a unidirectional trajectory down the Waddington landscape. Cellular potential becomes restricted as a totipotent zygote develops into a pluripotent mouse epiblast (or blastula in *Xenopus*), and finally into the lineage-specified (ectoderm, mesoderm, and endoderm) gastrula, defined by gradual loss of expression of the pluripotency factors. However, NCCs (arising from NPB in the ectoderm) seem to contradict this notion as they express pluripotency factors that endow them with broader developmental potential beyond their origin to give rise to the ectomesenchyme. A recent model suggests that the precursors of NCCs in the ectoderm reactivate the pluripotency network to reprogram and roll back up the Waddington landscape to give rise to ectomesenchyme. The reactivation model is an alternative, yet complementary update of an earlier model which suggested retention of pluripotency features in NCCs from the earlier embryonic stages that would promote multipotency.

The retention model is supported by studies that identified several NCC regulatory factors that played an important role in maintaining pluripotency in ESCs and NCC development, such as *Myc*, *Id3*, *Sox5*, *Tf-AP2*, *Ets1*, *FoxD3*, and *Snail1* in *Xenopus* (Bellmeyer, Krase, Lindgren, & LaBonne, 2003; Buitrago-Delgado, Nordin, Rao, Geary, & LaBonne, 2015; Cartwright et al., 2005; Light, Vernon, Lasorella, Iavarone, & LaBonne, 2005; Nordin & LaBonne, 2014; Ying, Nichols, Chambers, & Smith, 2003). In situ hybridization revealed that these genes were broadly expressed in the pluripotent blastula stage and were gradually restricted to the NPB (Bellmeyer et al., 2003; Buitrago-Delgado et al., 2015; Cartwright et al., 2005; Light et al., 2005; Nordin & LaBonne, 2014; Ying et al., 2003). Functional studies revealed that these factors maintained expression of the core pluripotency factors from the blastula (Buitrago-Delgado et al., 2015). When NCC transcription factors were overexpressed in early blastula explants, pluripotency was retained, as revealed by the ability of the cells to form mesoderm and interestingly, to endoderm as well. In contrast, overexpression in late explants had limited developmental potential (Buitrago-Delgado et al., 2015). Collectively, these results showed that the NCC lineage is specified before gastrulation and has greater plasticity beyond the ectoderm and the ectomesenchyme than previously appreciated.

Further supporting evidence for the retention model came from studies in various other model systems such as avian (chick; Basch, Bronner-Fraser, & García-Castro, 2006; Patthey, Edlund, & Gunhaga, 2009; Patthey, Gunhaga, & Edlund, 2008; Prasad, Uribe-Querol, et al., 2020), rabbit (Betters, Charney, & Garcia-Castro, 2018), and a human model based on differentiation of hESCs into NCCs (Gomez et al., 2019; Leung et al., 2016; Prasad, Charney, Patel, & García-Castro, 2020). Similar studies using explants showed that an intermediate region in the chick epiblast when dissected out and grown in neutral conditions displayed NCC fate specification, assessed by expression of NCC regulatory markers and a migratory phenotype (Basch et al., 2006; Prasad, Uribe-Querol, et al., 2020). These explants did not express neuroectodermal or mesodermal markers suggesting a direct origin from the pluripotent state (Prasad, Uribe-Querol, et al., 2020). A similar finding was observed in an in vitro neural crest differentiation model using hESCs (Gomez et al., 2019; Leung et al., 2016; Prasad, Charney, et al., 2020). Transcriptome analysis during differentiation revealed that within 6 hours of Wnt activation, there was a significant upregulation of NCC genes such as *PAX3*, *PAX7*, *MYB*, *ZIC3*, and *GBX2* (Gomez et al., 2019; Leung et al., 2016; Prasad, Charney, et al., 2020). Expression of many pluripotent genes was seen until 24 hr with very low expression of ectodermal genes, suggesting direct specification of NCC lineage from a pluripotent state (Gomez et al., 2019; Leung et al., 2016; Prasad, Charney, et al., 2020). While these studies provide evidence for NCC specification

before gastrulation, whether cells expressing NCC markers in the epiblast (or blastula) can give rise to terminally differentiated ectomesenchyme derivatives such as chondrocytes and osteocytes remains unclear.

The development of single-cell transcriptomics has allowed us to follow differentiation across various cell lineages of the embryo over time which has helped us better understand cellular heterogeneity in a more unbiased manner. Using single cell gene expression analysis, researchers profiled whole *Xenopus* embryos from the zygote stage to the tailbud stage and revealed that an intermediate stage of *Xenopus* development (between the blastula and the neural crest) had a neuroectoderm gene signature rather than a pluripotent-NCC signature (Briggs et al., 2018). These results suggested little retention of the pluripotency factors in cNCCs from the blastula stage (Briggs et al., 2018). Moreover, the low expression level of pluripotency genes postgastrulation was not limited to the ectoderm but was also present in mesoderm and endoderm, which would have been difficult to detect from in situ hybridization (Briggs et al., 2018). The low levels of transcripts detected for the pluripotency genes could be transcripts from earlier stages that may not have undergone degradation yet.

To address these contradictory findings, a recent study investigated whether the pluripotency program is reactivated or maintained from the pluripotent epiblast stage in cNCC precursors using a transgenic fluorescent reporter mouse and single-cell RNA-sequencing (Zalc et al., 2021). The authors found that precursors of cNCCs reactivated canonical pluripotency factors such as *Oct4*, *Nanog*, *Sox2*, and *Klf4*, with *Oct4* being one of the most enriched factors based upon transcriptomic data (Zalc et al., 2021). Using an *Oct4-GFP* reporter mouse, the authors observed that *Oct4* was highly expressed in the pluripotent epiblast stage of development (embryonic day E7.5) before downregulation, as the embryo became specified into different germ layers during gastrulation (E7.5–E7.75; Zalc et al., 2021). *Oct4* was then reactivated in the cNCC precursor cells (late E7.75, when the first two somites are formed), which gave rise to cNCCs that generated ectomesenchymal derivatives (Zalc et al., 2021). This finding was consistent with a study that examined the expression of various Oct4 homologs in *Xenopus* embryos during development (Morrison & Brickman, 2006). That study showed by in situ hybridization that *XIpou19* and *XIpou25* (Oct4) were de novo activated in the developing anterior neural tissue and posterior neural tube, as no transcripts were detected at intermediate stages (Morrison & Brickman, 2006). Loss of *Oct4* function in cNCCs caused craniofacial defects due to a decrease in proliferation and an increase in apoptosis of migratory cNCCs (Zalc et al., 2021). In contrast, cNCC-derived neurons and glia remained unaffected in *Oct4* mutants (Zalc et al., 2021).

Developmental cell fate decisions are accompanied by dynamic changes in chromatin. Pluripotency is associated with an open chromatin structure, which gradually acquires repressive histone and DNA marks, resulting in compacted chromatin and gene silencing in differentiated cells. During reprogramming, the unwinding of tight chromatin by exogenous factors promotes an epigenetic state that supports activation of the endogenous pluripotency program (reviewed in Apostolou & Hochedlinger, 2013). Comparison of accessible chromatin regions between *Oct4*+ cNCC precursors, mESCs, epiblast-like cells (EpiLCs), and epiblast stem cells (EpiSCs) revealed that the *Oct4*+ cNCC precursors resembled EpiSCs (Zalc et al., 2021). Open chromatin regions specific to cNCC precursors were associated with genes involved in NCC development and differentiation, glial cell differentiation, and cranial skeletal system development (Zalc et al., 2021). Interestingly, these genes were expressed at a lower level in *Oct4*+ cNCC precursors but increased later in delaminating and migratory NCCs (Zalc et al., 2021). Collectively, these observations suggest that an *Oct4*-centered pluripotency program is reactivated in mouse cNCC precursors to achieve two goals: (a) to open the locked chromatin state and expand the differentiation potential of cNCCs beyond the ectoderm; (b) to prime the chromatin landscape of cNCCs for future activation of migratory and differentiation programs (Zalc et al., 2021). However, one caveat of this study is the use of an *Oct4-GFP* reporter mouse which is an indirect measurement of OCT4 protein expression. Thus, the level of OCT4 protein present after *Oct4* transcriptional reactivation remains unknown. This is important because pluripotency factors associate as a complex to regulate the expression of their downstream target genes (Chew et al., 2005; Kuroda et al., 2005; Okumura-Nakanishi et al., 2005; Tomioka et al., 2002). Since the *Oct4-GFP* reporter mouse follows *Oct4* expression indirectly through the expression of GFP, it remains unclear whether OCT4 is associated with other pluripotency cofactors to regulate transcription in cNCCs.

Another recent study in *Xenopus* revealed that *ventx2* (Nanog) expression in NCCs is essential for the formation of ectomesenchyme (Scerbo & Monsoro-Burq, 2020). Consistent with the study in mice (Zalc et al., 2021), functional studies revealed that the gain of *ventx2* activity in late but not early gastrula stage embryos promoted NCC identity by enhancing expression of the NCC regulatory and pluripotency network genes in the NPB region (Scerbo & Monsoro-Burq, 2020). Together, these studies support the idea that NCCs have the potential to reprogram into a pluripotent state during development, and

TABLE 2 Summary of studies investigating expression of the pluripotency network genes during NCC development

Pluripotency genes	Model	Stage of development when expression of pluripotency genes was reported	Reference
Oct4, Nanog, and Klf4	Chick	HH9 stage (seven somites), cranial dorsal neural tube region	Lignell et al. (2017)
Oct4, Nanog, and Sox2	Human ESC differentiated into NCCs	First 24 hr of differentiation	Prasad, Charney, et al. (2020)
Oct4, Nanog, and Sox2	Mouse NCCs derived using Schwann cells- specific Cre (iris stromal region, craniofacial, and trunk region)	>E9.5 (in vitro sphere formation assay)	Hagiwara et al. (2014); Kikuchi et al. (2011)
Oct4, Nanog, and Sox2	Mouse trunk NCCs	E9.5 (NCCs migrated out from neural tube explants)	Fujita et al. (2016)
Oct4, Sox2, Klf4, and Trim71	Mouse cranial NCCs (neural tube explants and O9-1 cell line)	NCCs migrated from E10.5 neural tube explants and O9-1 cells cNCC line	Mohanty et al. (2016)
Oct4, Sox2, Nanog, Klf4	Mouse	Epiblast, NPB	Zalc et al. (2021)
PouV (Oct4), Ventx (Nanog), and Sox2	Xenopus	NPB (postgastrulation)	Morrison and Brickman (2006); Rogers et al. (2008); Scerbo et al. (2012)
PouV (Oct4), Ventx (Nanog), Sox2, Myc, Id3, Tf-AP2, Ets1, FoxD3, and Snail1	Xenopus	Blastula stage and NPB (postgastrulation)	Bellmeyer et al. (2003); Buitrago-Delgado et al. (2015); Cartwright et al. (2005); Light et al. (2005); Nordin and LaBonne (2014); Ying et al. (2003)
PouV (Oct4), Ventx (Nanog), Myc, Id3, Tf-AP2, Ets1, FoxD3, and Snail1	Xenopus	Blastula and NPB	Briggs et al. (2018)
Oct4	Xenopus	Anterior neural tissue and posterior neural tube	Morrison and Brickman (2006)
Ventx2 (Nanog)	Xenopus	Late gastrula stage	Scerbo and Monsoro-Burq (2020)

furthermore, reactivation of the pluripotency circuit is required to form the ectomesenchyme. It is worth noting that collecting early embryos with accurate staging (E7.5 vs. E7.75 vs. E8.0 in mouse/early vs. late blastula in *Xenopus*) can be challenging, thus, contributing to variability in some of the results presented in these studies.

Whether the pluripotency network is retained from an earlier stage or reactivated in NCCs remains controversial due to some of the technical challenges and species-specific differences (Table 2), which warrants further investigation (Figure 2). Nevertheless, it is now well established, across various species and using different investigative tools, that expression of the pluripotency factors in NCCs is essential for the formation of ectomesenchyme in vertebrates (Table 2). Oct4 has been shown to have a regulatory role in not just maintaining the pluripotent state but in orchestrating fate choice and tissue organization during development as well. A study showed that conditional deletion of *Oct4* in the epiblast cells resulted in the disruption of embryonic axis patterning and failure to differentiate into the germ layers (Mulas et al., 2018). Further investigation is required to determine the mechanisms that regulate the Oct4 pluripotency program in NCCs. Understanding how the pluripotency network regulates the expansion of differentiation potential of NCCs is likely to inform our understanding of common craniofacial birth defects. Finally, in the following section, we highlight research that would

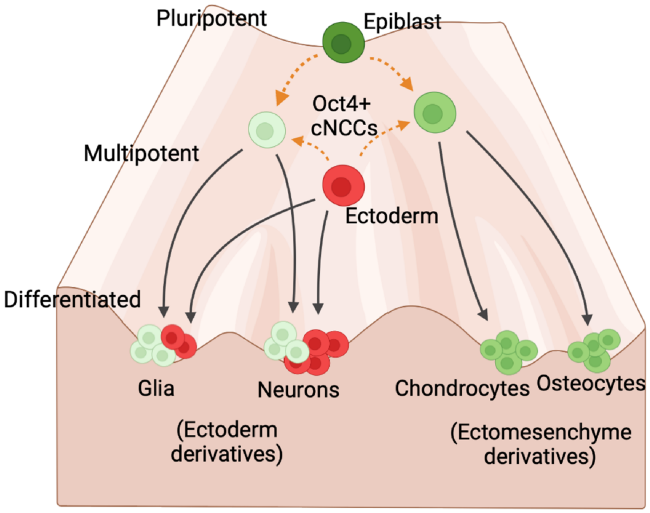

FIGURE 2 NCCs can follow alternate paths to express the Oct4-centered pluripotency program and expand their developmental potential (Adapted from Zalc et al., 2021). Waddington landscape depicting alternate paths that ectoderm cells may use to expand their developmental potential. Heterogeneity in *Oct4* expression may lead to a fate bias of NCCs into ectodermal or ectomesenchymal lineages

clarify the extent to which pluripotency regulatory mechanisms in stem cells can apply to NCC development.

2.3 | Common mechanisms regulate the pluripotency network in NCCs and ESCs

NCCs are specified in the ectoderm through concerted activities of different signaling pathways such as Wnt, BMP, Notch, and FGF signaling (reviewed in Simões-Costa & Bronner, 2015). These pathways also play an important role in governing naïve ESC versus primed EpiSC pluripotency states during early embryonic development as mentioned above (reviewed in Mossahebi-Mohammadi et al., 2020). However, the role of extrinsic signaling pathways in activating the NCC specification program and in regulating the pluripotency network in NCC precursors is incompletely understood. Assessment of chromatin accessibility in mouse embryos revealed that the chromatin landscape of *Oct4*+ cNCC precursors resembled that of EpiSCs (Zalc et al., 2021). The pluripotent state of mouse EpiSCs was shown to be governed by TGFβ and FGF signaling pathways (reviewed in Mossahebi-Mohammadi et al., 2020); whether these signaling pathways regulate *Oct4* transcription in NCC precursors needs further investigation. These pathways may bring signaling cues to the pluripotency network in NCCs to activate downstream NCC specification genes. *Oct4* expression is regulated by two enhancers: a distal enhancer and a proximal enhancer, which were differentially active in the inner cell mass and epiblast stages, respectively (Yeom et al., 1996). The question that then naturally arises is if the proximal enhancer has a dominant role in regulating *Oct4* expression in NCC precursors similar to EpiSCs.

Orphan NRs which bind to gene regulatory elements and recruit other factors to regulate gene transcription is relatively less well understood in NCCs. As mentioned previously, GCNF suppressed *Oct4* expression in pluripotent stem cells (Fuhrmann et al., 2001). Consistent with the gradual shutdown of pluripotency and onset of differentiation, *GCNF* was found to be expressed in all three germ layers of the late mouse epiblast stage (E7.5–E7.75; Chung et al., 2001; Dennis, 2008). Strong *GCNF* expression was observed in the dorsal neural folds, throughout the anterior neuroepithelium, at E8.5 (Chung et al., 2001; Dennis, 2008), consistent with dynamic changes in *Oct4* expression (Zalc et al., 2021). Several studies have analyzed *GCNF* expression during early mouse embryonic development (E7.5–E10.5); however, to date, no study has carefully looked at its expression in the anterior neural folds between the E7.75 and E8.0 stage, when the first two somites are formed. It is this late gastrula stage when *Oct4* and *ventx2* (Nanog) were shown to be reactivated in the NPB region in mouse and *Xenopus* embryos, respectively (Scerbo & Monsoro-Burq, 2020; Zalc et al., 2021). Additional studies are required to determine if *GCNF* levels are downregulated in the anterior neuroepithelium at the late gastrula stage to allow reactivation of the pluripotency network in NCC precursors.

Some groups have reported a positive role of *GCNF* in regulating differentiation of NCC progenitors (Dennis, 2008), which can be correlated with upregulation of *GCNF* expression in the anterior neural folds at E8.5 (Chung et al., 2001; Dennis, 2008). Loss of *GCNF* expression resulted in NCC-related defects (Dennis, 2008). *GCNF* mutants exhibited an expanded neural plate due to an increase in proliferation of neural progenitors and failure of NCCs to migrate from the neuroepithelium, suggesting that GCNF is required for the transition from precursors to migratory cNCCs (Dennis, 2008). These results are consistent with the gain of *ventx2* activity in late gastrula stage *Xenopus* embryos, which resulted in a lateral expansion of the NCC domain (Scerbo & Monsoro-Burq, 2020). Putative binding sites for GCNF were identified in the promoters of genes responsible for EMT in NCCs (Dennis, 2008). Altogether, these studies suggest that GCNF might have a role in coordinating exit from pluripotency and activation of pluripotent-NCC specification programs in NCC precursors. In contrast, LRH-1 positively regulated *Oct4* expression and was highly expressed in the pluripotent epiblast (Gu, Goodwin, et al., 2005); however, its expression pattern

during early somitogenesis in the anterior neural folds remains unknown. Further investigation is required to determine how orphan NRs such as GCNF and LRH-1 regulate the pluripotency network to initiate NCC specification programs.

The distal *Oct4* enhancer which is active in the naïve pluripotent state may also have a role in *Oct4* regulation in NCC precursors. Sall4, a pluripotency transcription factor, is highly expressed in ES cells and activates *Oct4* by binding to its distal enhancer element (Zhang et al., 2006). Neuromesodermal progenitors (NMPs) give rise to neural and paraxial mesodermal progenitors in the trunk and tail during embryonic development (Tahara et al., 2019). Loss of *Sall4* in NMPs led to accelerated differentiation of NMPs toward the neuronal lineage at the expense of presomitic mesoderm (Y. Wang et al., 2013). *Sall4* mutants displayed developmental defects such as a truncated tail and disorganized vertebrae (Tahara et al., 2019). Some of the trunk NCCs which are biased toward neuronal fate were shown to arise in zebrafish from posterior NMP-neural cells in the tailbud (Lukoseviciute, Mayes, & Sauka-Spengler, 2021). It will be interesting to see if NMPs that give rise to trunk NCCs express different levels of *Sall4* compared to the rest of the NMP population. Strong *Sall4* expression was seen in craniofacial structures such as the frontal nasal structure, lower jaw, and first branchial arch between E8.5 and E10.5 (Kohlhase et al., 2002; Tahara, Kawakami, Zhang, Zarkower, & Kawakami, 2018). However, the role of Sall4 in the development of these structures remains poorly understood. Thus, Sall4 is a candidate factor to regulate *Oct4* in NCCs and merits further investigation. However, it is to be noted that *Sall4* expression in NMPs, and craniofacial structures may just be a progenitor marker and have nothing to do with the regulation of the pluripotency network.

Along with changes in gene expression and epigenetic profile, expression levels of miRNAs also change during reprogramming (Polo et al., 2012). OSK-induced reprogramming of MEFs resulted in stochastic activation of two pluripotent miRNA clusters, *miR-302* and *miR-290* (Parchem et al., 2014). In contrast, OSK + Sall4-mediated reprogramming induced a uniform sequential activation of *miR-302* followed by *miR-290* during late stages of somatic cell reprogramming and a 10-fold increase in reprogramming efficiency (Parchem et al., 2014). These results suggest that somatic cells can follow alternative paths during reprogramming, governed by the expression of transcription factors and miRNAs. This paradigm may also extend to the regulation of the pluripotency program in NCCs. The *miR-302/367* cluster of miRNAs can reprogram somatic cells into iPSCs without any additional expression of transcription factors (Anokye-Danso et al., 2011). *Oct4* and *miR-302* are co-expressed in the epiblast during early embryonic development and in ESCs (Card et al., 2008). *MiR-302* was broadly expressed in the embryo at the epiblast stage until E9.5 (Parchem et al., 2015, 2014) and remained expressed in cNCCs during specification, delamination, and migration (Parchem et al., 2015). Since *miR-302* positively regulated *Oct4* expression in hESCs (H. L. Li et al., 2016), *miR-302* may have a role in *Oct4* regulation in cNCC precursors as well. Moreover, OCT4, SOX2, and NANOG were shown to transcriptionally regulate *miR-302* expression by binding to the promoter region of the *miR-302* cluster in hESCs (Card et al., 2008).

Lin28a is another pluripotency factor that was strongly expressed in the cranial neural folds, premigratory and early migratory NCCs in the chick embryo (Simoes-Costa & Bronner, 2016; Simões-Costa, Tan-Cabugao, Antoshechkin, Sauka-Spengler, & Bronner, 2014). *Lin28a* was known to be part of the pluripotency network that maintains stemness and reprograms somatic cells into iPSCs (Yu et al., 2007; Zhang et al., 2016). *Lin28a* was shown to be involved in maintaining NC stem cell identity and promoting NCC multipotency in chick embryos (Bhattacharya, Rothstein, Azambuja, & Simoes-Costa, 2018). Expression of *Lin28a* promoted differentiation of NCCs into multiples cell types, such as neurons, glia, melanocytes, chondroblasts, and smooth muscle cells (Bhattacharya et al., 2018), suggesting a role for *Lin28a* in maintaining stem cell properties of NCCs. *Lin28a* has been shown to regulate genes posttranscriptionally by inhibiting the *let-7* family of miRNAs (Newman, Thomson, & Hammond, 2008). Functional studies showed that *lin28a* regulated NCC development in a *let-7* dependent manner (Tahara et al., 2019). Disruption of the *lin28a-let-7* axis by loss of *Lin28a* and gain of *let-7* resulted in downregulation of NCC and stem cell genes that contained *let-7* target sites (Bhattacharya et al., 2018). *Let-7* was also shown to form a feedback regulatory loop with *Lin28a* (Bhattacharya et al., 2018). Furthermore, the enhancer that drove the expression of *Lin28a* in neural crest cells contained four TCF/LEF (downstream factors of Wnt signaling pathway) binding sites (Bhattacharya et al., 2018). Disruption of the binding sites resulted in complete loss of enhancer activity and knocking down Wnt1 and Wnt4 resulted in decreased *Lin28a* expression and increased levels of mature *let-7* (Bhattacharya et al., 2018). Together, these results suggest that the pluripotency factor *Lin28a* maintains multipotency in NCCs by preventing *let-7* mediated repression of the pluripotency network regulated by Wnt signaling. These studies suggest that NCCs may co-opt autoregulatory feedback circuitry of pluripotent transcription factors, miRNAs, and signaling pathways to

regulate the pluripotency network and expand their developmental potential (Table 1).

As development progresses and NCCs migrate and differentiate into different cell types, the pluripotency program must be shut off, as it can lead to aberrant tumor formation. Interestingly, overexpression of *miR-145*, which directly targeted *OCT4*, *SOX2*, and KLF*4* in hESCs, resulted in differentiation of hESCs into ectoderm and mesoderm lineages and not endoderm or trophectoderm lineage (Xu et al., 2009). Hence, we speculate that *miR-145* might be involved in downregulating *Oct4* transcript levels in NCCs as they delaminate and migrate to give rise to ectodermal and ectomesenchymal derivatives. Further investigation is needed to determine the miRNA profile of NCCs at a single-cell resolution, and how the pluripotency network can be regulated by different miRNAs during NCC development.

3 | CONCLUSION

This review highlights some of the mechanistic programs that may be co-opted by NCCs during development, to regulate the pluripotent state and expand their developmental potential. Several factors may synergistically or antagonistically regulate *Oct4* expression in NCCs by feedback regulation, such as epigenetic modifiers, noncoding RNAs, signaling factors, and transcription factors. Transcriptional heterogeneity of NCCs is believed to be important for expanded developmental potential and later diversification and differentiation into various cell types. Thus, it will be important to investigate the interplay between pluripotency network genes and regulatory factors mentioned in this review in individual cells. One key question is whether different NCCs express different levels of the core pluripotency factors and if these factors coregulate the same or different sets of target genes to control heterogeneity. Pluripotency transcription factors and miRNAs expressed in NCCs may also form a feedback regulatory loop and co-regulate downstream lineage-specific genes. Further investigation is required to determine the crosstalk between the pluripotency state and fate specification in NCCs at single-cell resolution. Lastly, chromatin accessibility remains a major barrier for reactivation of the pluripotency program and efficient somatic cell reprogramming. Future studies are required to examine the heterogeneity of expression of epigenetic machinery in NCCs and how these modifiers differentially regulate histone modifications at NCC lineage specification genes. These studies can determine whether the ectodermal and ectomesenchymal lineage genes are differentially regulated in *Oct4*+ NCCs by chromatin accessibility. Indeed, differences in histone modifications can reflect the timing of expression of lineage-specific genes resulting in cell fate bias toward ectodermal or ectomesenchymal lineages in NCCs. Further investigation into these mechanisms will provide exciting new insights into reprogramming and NCC development. These studies will further our understanding of how reprogramming of cNCCs can be utilized as a therapeutic tool for treating and preventing patients with craniofacial defects.

ACKNOWLEDGMENTS

This study is supported by the National Institutes of Health R01-HD099252, R01-HD098131 (Ronald J. Parchem), CPRIT Scholar in Cancer Research RR150106 (Ronald J. Parchem), Andrew McDonough B+ Foundation (Ronald J. Parchem), and V Scholar in Cancer Research #V2017-017 (Ronald J. Parchem) grants. The authors would like to thank all the lab members, Dr Andrew Groves, Dr Bruno Di Stefano, Dr Kyle Eagen, and Dr Jacqueline G. Parchem for their suggestions and critical feedback on the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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How to cite this article: Patel, I., & Parchem, R. J. (2022). Regulation of *Oct4* in stem cells and neural crest cells. *Birth Defects Research*, *114*(16), 983–1002. https://doi.org/10.1002/bdr2.2007