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Molecule of the month

NaNog: A pluripotency homeobox (master) molecule

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ABSTRACT

One of the most intriguing aspects of cell biology is the state of pluripotency, where the cell is capable of self-renewal for as many times as deemed “necessary”, then at a specified time can differentiate into any type of cell. This fundamental process is required during organogenesis in foetal life and importantly during tissue repair in health and disease. Pluripotency is very tightly regulated, as any dysregulation can result in congenital defects, inability to repair damage, or cancer. Fuelled by the relatively recent interest in stem cell biology and tissue regeneration, the molecules implicated in regulating pluripotency have been the subject of extensive research. One of the important molecules involved in pluripotency, is NaNog, the subject of this article.

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INTRODUCING 'NANOG'

'And our souls were young again in Tir Na Nog' sings the Irish songwriter and musician Van Morrison in one of his famous songs, referring to the Irish Legend of Oisín and Niamh and the Land of Eternal Youth, beauty, health and joy - Tír Na nÓg (Fig. 1). Inspired by the land of Tír Na nÓg, the divergent homeobox transcription factor that plays a crucial role in embryonic stem (ES) cell fate specification and self-renewal was coined Nanog. In 2003, Nanog was introduced in two papers published in the same issue of *Cell* and was celebrated as the 'new recruit to the ES cell orchestra'.¹⁻³

Until its discovery, knowledge of what defines the potency of mouse embryonic stem cells revolved around a quartet of transcription factors; Oct4, Sox2, FoxD3, and Stat3.

Oct4 is required for cell fate regulation in the early embryo, is expressed in the inner cell mass (ICM), and is down regulated upon differentiation into trophoblast cells.⁴ Sox2 and FoxD3 are known to be involved later in the maintenance of the epiblast after implantation.^{5,6} Stat3 activation by the cytokine Leukemia Inhibitory Factor (LIF) is required to sustain self-renewal of cultured ES cells.⁷⁻⁹

Chambers *et al.* and Mitsui *et al.* did not only describe the homeobox transcription factor Nanog and its expression in the inner cells of a compacted morula, blastocyst, early germ cells and in the ES and embryonic germ (EG) cell lines derived from these stages, they also showed how embryo cell fate specification and ES cell self-renewal may be related.

THE VARIANTS OF NANOG

The Nanog gene is related to the NK homeobox genes first described by Kim & Nirenberg in *Drosophila*¹⁰ and shows close sequence alignment with the transcription factors Msx1, Nkx2.5 and Barx1 (50% amino acid identity in the protein's homeodomain). NANOG promoter region, 299 bp large



Figure 1. The Irish Legend of Oisín and Niamh and the Land of Eternal Youth, beauty, health and joy - Tír Na nÓg: [...] As the beautiful woman and her horse drew nearer, all the men (great warrior Oisín with his father the legendary Finn MacCool the leader of the Fianna – a group of great protectors who guarded the High King of Ireland) stopped in their tracks, waiting to hear what she had to say. “My name is Niamh,” said the goldenhaired maiden, “my father is the King of the mystical land of Tír Na nÓg, a land that knows no sorrow and where nobody ever ages. I have heard wonderful things of a great warrior named Oisín, and I have come to take him with me back to the Land of Eternal Youth.” Oisín immediately fell in love with Niamh and agreed to join Niamh on horseback to go and live in Tír Na nÓg, promising his father that he would return to Ireland to see him again soon.[...] Source: <http://irelandofthewelcomes.com>

(- 264 to +35) upstream of exon 1 contains five CpG-dinucleotides, which are subjected to DNA-methylation¹¹. This NANOG promoter region also contains an OCT3/4-SOX2 binding motif, a TATA-box and binding sites for the transcription factors AP-2, SP1 and TFIIID.^{11,12} There are four transcript variants of the mRNA encoded by NANOG: Transcript variants NANOG-001 (2101 bp mRNA) and - 002 (870 bp mRNA) are known as protein coding, while variant - 004 (561 bp mRNA) is putatively protein coding and variant - 003 is known as nonsense mediated decay. NANOG transcription can be regulated by binding of OCT3/4 and SOX2 to their binding motifs in the NANOG promoter.¹¹⁻¹³ Eleven Nanog pseudogenes - a large number for a homeobox gene (more details on this are described in the next paragraph) - have been described in the human genome.¹⁴ Ten are processed (NANOGP2 - NANOGP11) and one is a non-functional duplication (NANOGP1) genome.

IT'S A HOMEBOX (NOT A HOX) GENE

In humans, the NANOG gene is located on the short arm of chromosome 12 (12p13.31) from 7940390 - 7948655 and encompasses 8265 bp of DNA. NANOG is a homeobox gene. Homeobox genes are a group of genes with a common DNA sequence that are involved in body segmentation and cell differentiation during embryonic development. They contain a 180-base-pair segment (the "homeobox") that encodes a 60 amino acid DNA-binding protein domain called the homeobox domain or homeodomain. The homeodomain binds DNA and acts as a transcription factor that regulates expression of a number of target genes. It was first identified in a number of homeotic and segmentation proteins in the fruit fly *Drosophila Melanogaster*.^{15,16} In 1995 the Nobel Prize in Physiology or Medicine was awarded to Ed Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus for the identification and classification of 15 genes of key importance in determining the body plan and the formation of body segments of *Drosophila*, the Hox genes. "Homeotic mutations" of the Hox genes

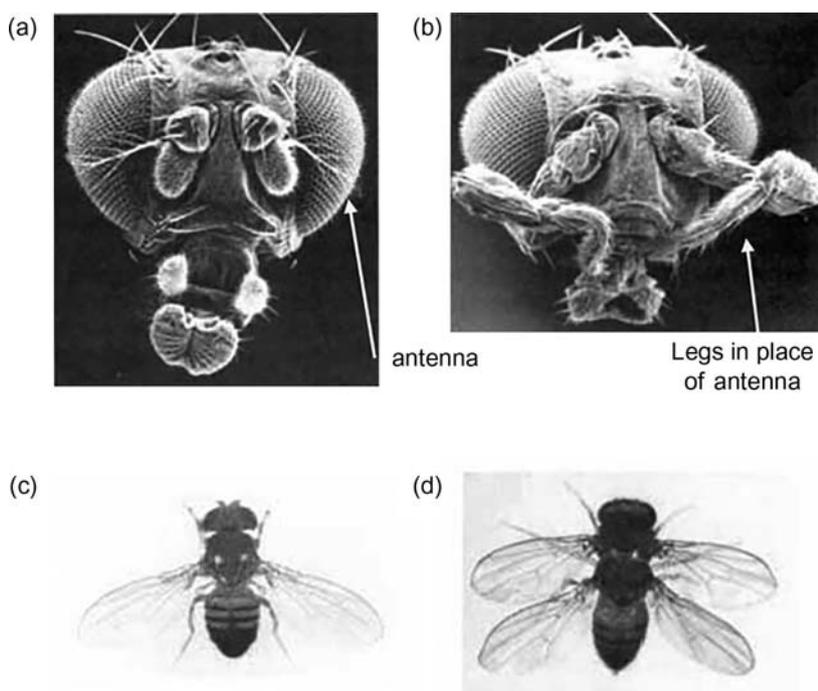


Figure 2. The effects of mutations in homeotic genes. The first Homeobox genes discovered were *Drosophila melanogaster* developmental control genes that specify the identity of each body segment by controlling the identity of the organs that develop within that segment, the HOX genes. They were described as "homeotic" genes. "Homeotic" is a functional description for genes that cause homeotic transformations. All homeotic genes are transcriptional regulators. In *Drosophila* numerous 'homeotic transformations' were observed, where e.g. body parts seem to have been replaced by other. Known homeotic mutations cause the formation of an extra set of wings, or two legs at the position in the head where the antennae of a *Drosophila* are normally found. (a) Normal *Drosophila* head. (b) *Drosophila* homeotic mutant (Antennapedia): antennae are replaced by legs. (c) Normal *Drosophila* body structure. (d) Homeotic mutant (Bithorax): segment has developed incorrectly to produce an extra set of wings. Images are courtesy of the Archives, California Institute of Technology.

cause “homeotic transformations”, where for example body parts seem to have been replaced by other (Fig. 2). Even though the homeodomain binds DNA in a sequence-specific manner, homeodomain proteins mostly act in the promoter region of their target genes as complexes with other transcription factors, which provides higher target specificity than the homeodomain protein alone. Homeodomains are encoded by different clusters of Homeobox genes, e.g. the Hox-, the ParaHox- and the NK gene clusters.

NANOG'S SIGNATURE STRUCTURE

NANOG-001 (referred to as Nanog_human or Nanog in this review) is a 305 aa long protein with a molecular weight of 34.6 kDa usually analyzed to study the role of NANOG. It is a multidomain protein with a well-conserved Nk-2 homeodomain.^{1,2,17,18} N-terminal region contains 95 residues rich in Ser and Thr and acidic residues found in typical transactivators.^{1,2,17} The signature homeodomain spans from aa 95-155 and facilitates DNA-binding and gene regulation and interaction with other proteins.^{17,19,20} Formation of secondary structures (helix, strand and turn) occurs mainly within the homeobox-coding region. C-terminal to the homeodomain, Nanog contains a tryptophan repeat (WR) domain in which every fifth residue is a tryptophan. Reports from two laboratories reveal that the WR domain mediates Nanog dimerization and is required to confer LIF-independent self-renewal in ES cells.^{21,22} A Nanog mutant with an alteration of 20 tryptophans to alanines within the WR domain lost the capacity to interact with several other pluripotency network proteins, including Sall4, Zfp281, Zfp198 and Dax1.²² The WR domain is conserved in the placental mammalian orthologues of Nanog. The NANOG protein is unstable with a half-life of 120 min in human ES cells, a characteristic of proteins involved in tightly controlled interaction networks.²³

THE ROLE OF NANOG IN MAMMALIAN EMBRYOLOGY

Embryonic development in a nutshell

The development of the mammalian embryo involves a series of cleavage divisions and cell fate determinations that are tightly regulated at each developmental stage. For example, the transition from the compacted morula to the early blastocyst stage is accompanied by a strict separation of two cell lineages of the embryo, namely the trophoectoderm, which is the extra-embryonic structure that gives rise to the trophoblast lineage, and the inner cell mass (ICM), which produces all embryonic tissues. Contrary to the committed cells of the trophoectoderm, cells of the ICM are pluripotent and hence are able to generate ES cells in vitro or develop into the embryo founder tissue, known as the epiblast, in vivo.^{24–26} In addition, a subset of the ICM cells differentiate into another extra-embryonic layer referred to as the primitive endoderm to further support the developing embryo.

Nanog “masters” the specification of ES cell identity

In an attempt to develop a deeper understanding of embryonic development, research studies have focused on uncovering key genes that are involved in lineage specification and pluripotency. As mentioned earlier, loss-of-function studies have identified Nanog as the main player in maintaining the pluripotency of the ICM in vivo, self-renewal of ES cells in vitro as well as driving the development of the subsequent pluripotent epiblast into the germ cell lineage.^{1,2}

The significant role of Nanog in maintaining pluripotency was highlighted when the main players in pluripotency, Oct3/4, Sox2 and Stat3, failed to support LIF-independent self-renewal in ES cells.^{1,2} Both ES cells in vitro and pluripotent epiblast cells in vivo require both Oct3/4 and Nanog to evade differentiation into extra-embryonic lineages and additional factors such as LIF-activated Stat3 prolong their pluripotent potential. In the absence of Oct3/4, ES cells lose their pluripotent capacity and differentiate into the trophoectoderm.²⁷ Surprisingly, Niwa et al. have shown that elevating the expression of Oct3/4 in ES cells beyond the endogenous level triggers their differentiation into the primitive endoderm.²⁸ Therefore, these conflicting roles of Oct3/4 suggest that it plays a significant role in cell fate determination by tightly regulating a broad range of genes during the early stage of embryonic development. As opposed to Oct3/4, Nanog has been shown to maintain the pluripotency of ES cells even in the absence of the LIF/Stat3 pathway.^{1,2} In the absence of LIF, Oct3/4 is unable to prevent ES cells from differentiating into the trophoectoderm lineage and even elevating Oct3/4 levels does not rescue pluripotent ES cells from reverting back to a differentiated state. Increasing the expression of Nanog, on the other hand, circumvents the need for LIF/Stat3 expression to block ES cells

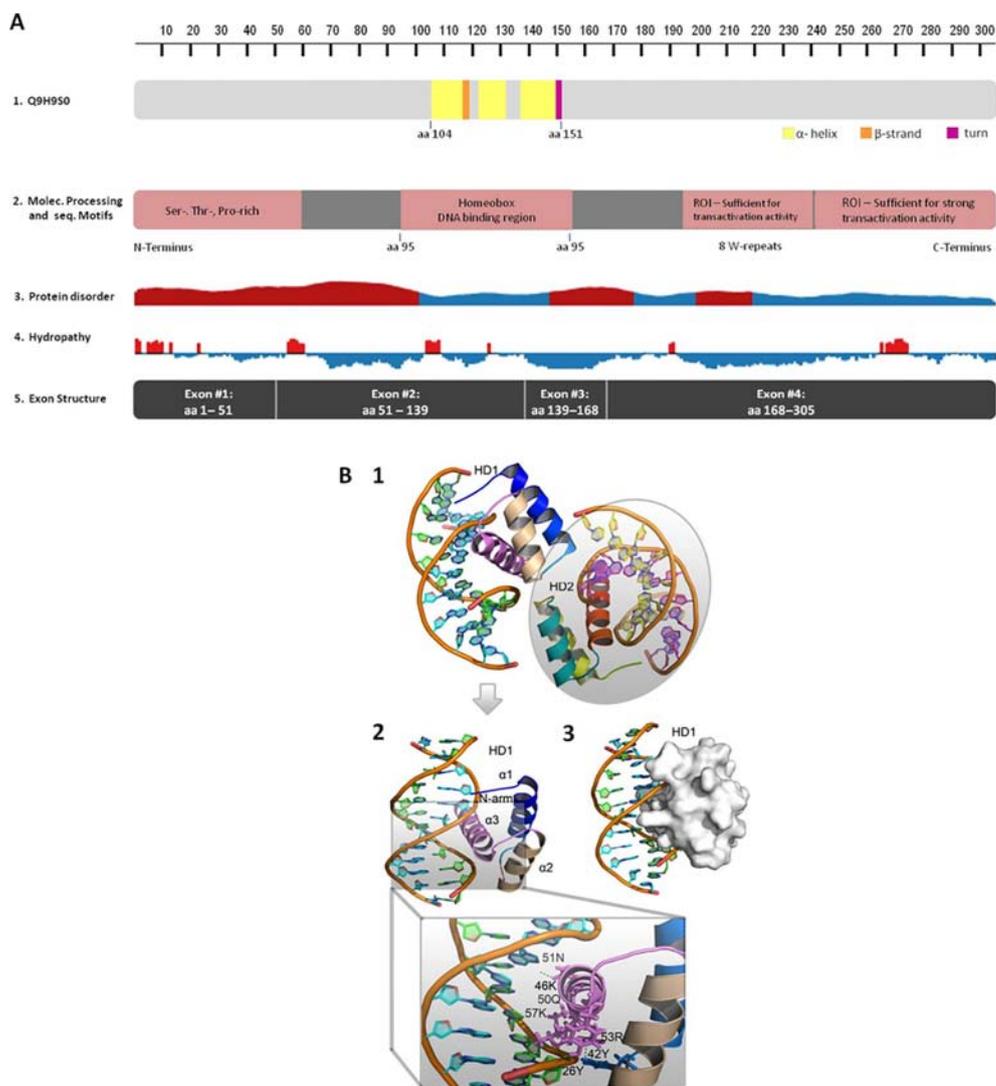


Figure 3 (A). 1) UniProtKB sequence of NANOG_HUMAN with secondary structure motifs, Length 305 aa. 2) UniProtKB chains with UniProtKB sequence motifs. 3) Predicted regions of protein disorder based on Ironn (Troshin, P. and Barton, G. J. unpublished), a Java implementation of RONN (Ref). Red: probably disordered, Blue: probably ordered. 4) Hydropathy has been calculated using a sliding window of 15 residues and summing up scores from standard hydrophobicity tables. Red: hydrophobic, Blue: hydrophilic. 5) Exome boundaries mapped from chromosomal coordinates onto UniProt Sequence. Figure adapted from RCSB PDB Data base. Data Source: 1, 2 and 3: UniProtKB, 4 and 5 were calculated using BioJava. (B) Molecular models of Nanog homeobox. 1) A 3D cartoon illustration of dimer state of Nanog homeodomain (HD) with DNA strands. 2) The monomer model of HD with DNA and the extended figure panel at the bottom shows a close-up view of the key residues at their interface. 3) The surface (white) map of monomeric HD provides an external interaction pattern of the complex. All the models are created using the 3D data of crystal structure of a nanog homeobox from human and the data was retrieved from protein data bank (ID: 4RBO). The molecular artworks are prepared with PyMOL.

differentiation into the primitive endoderm and supports ESC self-renewal.² These findings define Nanog as a “master” player in the specification of ES cells identity, superseding the roles of Oct3/4 and LIF/Stat3 in maintaining pluripotency (Fig. 4).

Nanog is a crucial pluripotency-sustaining factor in the pre-implantation embryo

But how does Nanog determine cell fate in the ICM of the pre-implantation mammalian embryo? A study by Loh et al., 2006 has shown that Nanog-deficient mouse embryos tend to generate pluripotent cells in the initial phase of development but then suddenly differentiate into the primitive endoderm.²⁷ This indicates that the effect of Nanog in maintaining pluripotency becomes crucial at a later stage in embryonic development after Oct3/4 has regulated cell fate in the initial phase. In fact, Mitsui et al.,

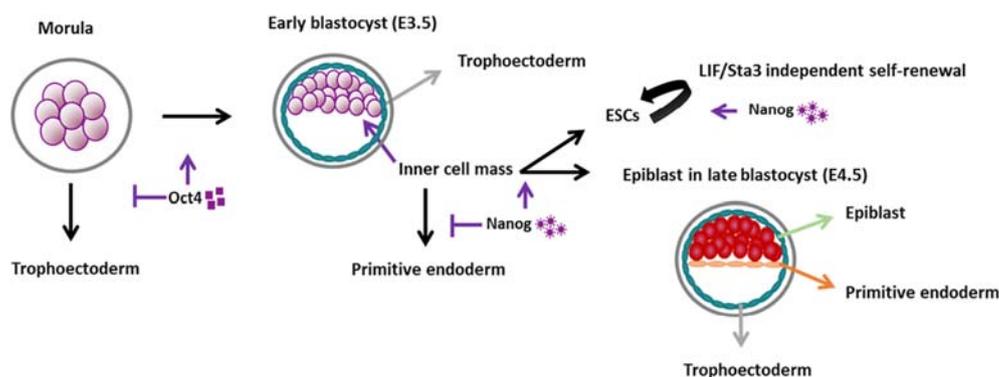


Figure 4. The contribution of Nanog, Oct3/4 and LIF/Stat3 during embryonic development. Oct3/4 facilitates the development from the morula into the early blastocyst stage (E3.5) by inactivating trophoectoderm differentiation. Nanog mediates the transition of pluripotent inner cell mass (ICM) cells into epiblast cells between E3.5 and E4.5 in vivo. Additionally, Nanog-expressing ICM cells are able to generate pluripotent ESCs in vitro. Nanog supports ESC self-renewal in a LIF/Stat3 independent manner.

2003 showed that Oct3/4 tends to be highly expressed whereas Nanog levels remain low during the early stage of embryonic development; specifically at the morula stage.² Similar to ES cells, the expression of Oct3/4 at this stage is critical for maintaining pluripotency. The expression pattern of Nanog changes dramatically as the pre-implantation embryo progresses into the early blastocyst stage (E3.5), the point at which Nanog becomes the key determinant of cell fate.² While Oct3/4 expression is more broadly expressed in the mouse embryo at E3.5, Nanog expression is restricted to the subset of ICM cells that will eventually give rise to the pluripotent epiblast during the late blastocyst stage (E4.5).^{24,29,30} The remaining cells of the ICM that do not express Nanog have been shown to exclusively express endoderm-specific genes, such as Gata4/6, which drive their differentiation into the primitive endoderm at E4.5.^{25,31} Taken together, these findings identify Nanog as the major lineage specification factor maintaining the pluripotency of ICM cells throughout the E3.5 to E4.5 transition.

Nanog and chromosome X-reactivation

Female mammals have two gene-rich X chromosomes whereas the male has a single X chromosome and a Y chromosome, which carries a limited amount of genes.³² If both X-linked genes were equally expressed, females would express twice as many X-linked genes as would males creating an imbalance between both sexes. Therefore, female placental mammals have developed a strategy of X-chromosome inactivation (XCI) to resolve the X-linked dosage discrepancy between both sexes. The mechanism of XCI enables one of the two X chromosomes in the female to be transcriptionally silenced in a completely random manner; that is, both maternal and paternal X chromosomes have an equal chance of being inactivated.^{33,34} However, at what stage of embryonic development does the X chromosome become silenced and how is it regulated? Studies performed on mouse ES cells, in which the mechanism of XCI can be recapitulated, have revealed new insights into this unique phenomenon.³³

XCI takes place initially at the early blastocyst stage (E3.5) where the paternal X chromosome is preferentially silenced.^{34,35} The progression into the late blastocyst stage (E4.5), however, is accompanied by a reactivation of the silenced paternal X chromosome specifically in the pluripotent epiblast in order to subsequently allow either the maternal or paternal X to be randomly inactivated in the female embryo.^{34,36} Trophoblast cells, on the other hand, retain the inactivated form of paternal X chromosome throughout embryonic development.^{25,34} These results suggest that X-reactivation could serve as a marker for ground state pluripotency. In fact, studies have shown that at E3.5, the subset of ICM cells expressing Nanog - the “master” gene of pluripotency - are able to reactivate the silenced paternal X-chromosome as they progress into E4.5.²⁵ Expectedly, the subset of Nanog-negative ICM fail to reactivate the paternal X-chromosome at E3.5, which explains why cells of the primitive endoderm and trophoectoderm lineage retain the silenced form of the paternal X chromosome throughout development.²⁵ Hence, these findings suggest that Nanog couples pluripotency of epiblast cells with X-reactivation; however, the exact mechanisms require further investigation.

A “master gene” for molecular reprogramming?

Similar to pluripotent epiblast cells, reprogrammed induced pluripotent stem cells (iPSCs), the subject of this section, re-activate the paternal X chromosome.³³ Hence, X-reactivation could serve as a marker for pluripotency. The discovery of iPSCs was initiated by Takahashi and Yamanaka in 2006 when they reported a revolutionary approach in the field of stem cell biology whereby differentiated cells could be re-programmed into a pluripotent state via exogenous expression of 4 transcription factors (Oct4, Sox2, Klf4 and c-Myc).³⁷ Sharing similar morphological and functional properties with ES cells, iPSCs have opened up new avenues for regeneration therapies. Surprisingly, Nanog was not included in the quartet of pluripotency-promoting transcription factors. However, ensuing studies suggested that the function of Nanog during the reprogramming process might be analogous to its crucial role in establishing pluripotency in ICM cells.²⁵

Nanog converts pre-pluripotent cells into fully reprogrammed iPSCs

Studies have shown that Nanog-deficient ICM cells fail to develop into epiblasts and are unable to give rise to ESCs. Instead, they are locked in a pre-pluripotent state leading to apoptotic behavior.^{2,25,38} Knock down of Nanog in established ES cells, on the other hand, does not alter their self-renewal capacity or their ability to differentiate into germ cell lineages.¹ Similarly, Nanog-deficient pre-iPSCs fail to achieve a pluripotent ground state and are non-viable, whereas deletion of Nanog in fully reprogrammed iPSCs has no effect on their pluripotent potential.^{39,40} These findings suggest that Nanog’s role during molecular reprogramming could be dispensable during the initiation process; which is achieved by the quartet to generate partially reprogrammed pre-iPSCs, but later becomes crucial to produce bona fide iPSCs (Fig. 5).^{40,41} In fact, a study by Lee et al., 2013 showed that miR-302-mediated knock down of methyl-DNA binding domain protein 2 (MBD2), which transcriptionally inactivates Nanog, significantly enhances the reprogramming efficiency of pre-iPSCs by increasing Nanog expression.

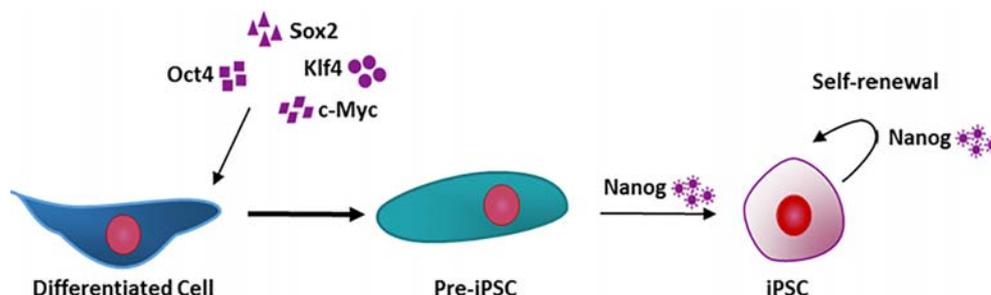


Figure 5. Requirement of Nanog during the reprogramming process. Partial reprogramming of a differentiated cell into a pre-iPSC is achieved by the exogenous expression of the reprogramming factors (Oct4, Sox2, Klf4, c-Myc). Nanog is dispensable at this stage. The conversion of pre-iPSCs into bona fide iPSCs is dependent upon Nanog expression. Inactivation of Nanog at this stage does not alter the self-renewal capacity of iPSCs.

Nanog cooperates with reprogramming factors and epigenetic regulators to achieve ground state pluripotency

How does Nanog facilitate the transition of pre-iPSCs into ground state pluripotency? Chromatin immunoprecipitation studies have shown that in fully reprogrammed iPSCs Nanog forms protein complexes with Oct4, Sox2 and Klf4 at promoter targets that are also occupied by Nanog in ES cells.^{42–44} Therefore, the transition from the pre-iPS to the iPS state requires Nanog-mediated recruitment of the reprogramming factors to promoters of the pluripotent genes or Nanog-induced epigenetic remodelling of these sites rendering them accessible to the exogenous transgenes. These findings suggest that Nanog acts as a “master gene” orchestrating the molecular switch to a purely undifferentiated state. Interestingly, recent evidence has revealed new insights into the role of Nanog in reprogramming. For example, Schwarz et al., 2014 reported that minor changes in iPS culture conditions could circumvent the need for Nanog to produce bona fide iPSCs.⁴⁵ The authors were able to show that the addition of ascorbic acid to iPS medium facilitates the transition of Nanog-deficient mouse embryonic fibroblasts (MEFs) into fully reprogrammed iPSCs that functionally resembled wild-type iPSCs. This finding suggests that iPSC generation could be achieved via distinct yet

redundant pathways. Therefore, further studies are warranted to elucidate the different mechanisms by which fully reprogrammed iPSCs could be generated.

Conclusion and future directions

Successful translation of the recent advances in stem-cell research to the clinical arena depends profoundly on careful understanding of the complex biological networks governing cell repair and regeneration. Elucidating the role of master molecules, i.e. molecules that regulate mass gene transcription such as Nanog, is a crucial step in deciphering these networks. While our understanding of Nanog's role in embryogenesis has grown tremendously over the past few years, further research on its role in inducing and maintaining pluripotency in adult life is needed. Until then, the quest for T_rNa nOg continues.

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