**Embryonic Stem Cell Markers: An Overview**

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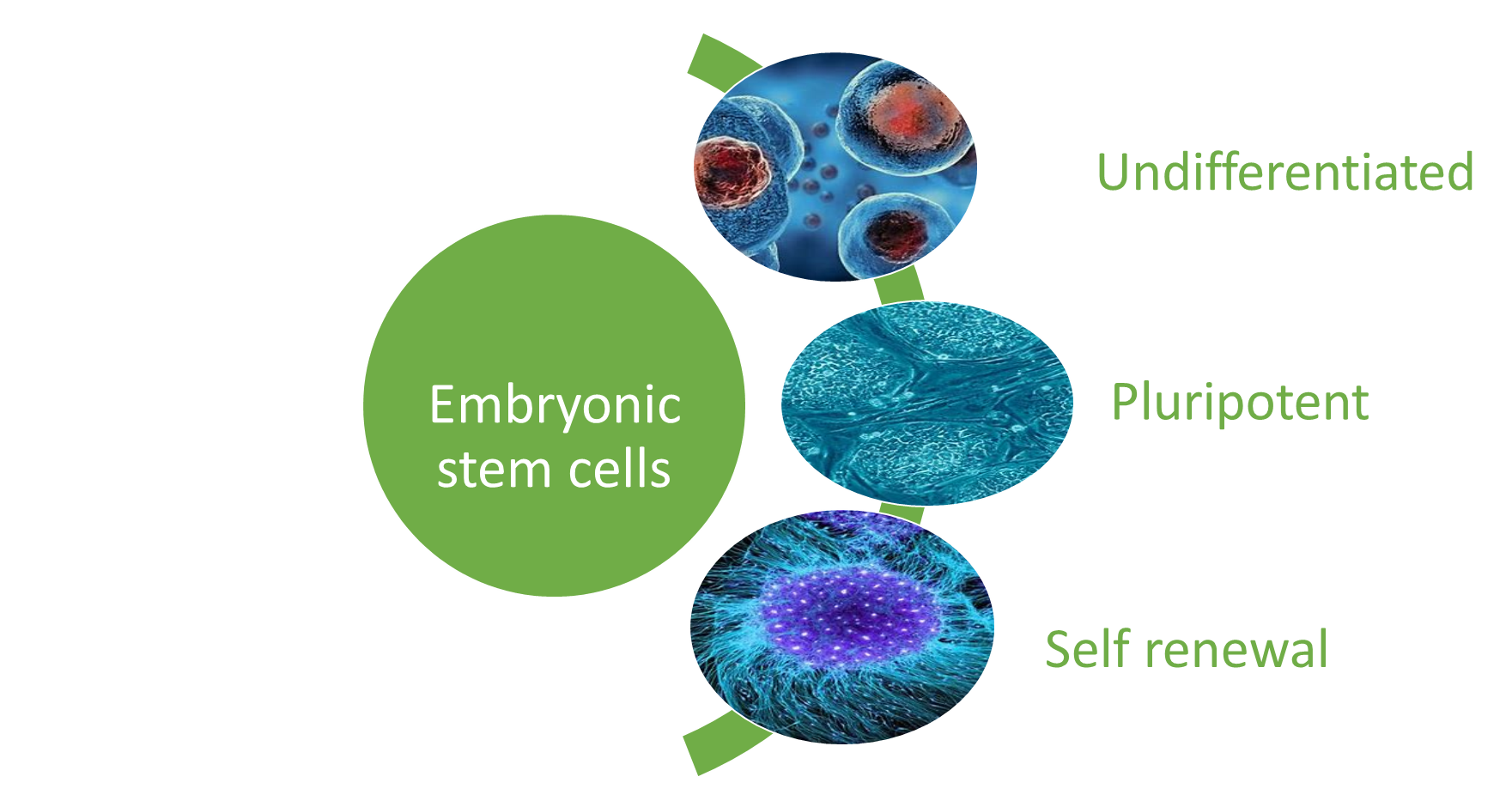
**Abstract**

Embryonic stem cell (ESC) markers are molecules specifically expressed in ES cells. Understanding their functionality is crucial for characterizing and clarifying ESC pluripotent maintenance and self-renewal mechanisms, facilitating clinical applications of ES cells. Separating ES cells from other cell types, especially tumor cells sharing similar markers, poses a fundamental therapeutic challenge. To address this, a comprehensive manufacturer list aids in identifying and extracting ESCs using marker-based flow cytometry (FCM) and magnetic cell sorting (MACS), the most efficient cell isolation techniques available. Here, we discuss various cell surface and generic molecular markers indicative of undifferentiated ESCs. Additionally, it summarizes other molecules like lectins and peptides that bind to ESCs via affinity and specificity. The review also explores markers overlapping with tumor stem cells (TSCs), raising uncertainty about their individual or combined use in cell identification and isolation. This chapter presents ESC markers, including cell surface and generic molecular markers, as well as lectins and peptides. The complexity of marker selection is highlighted, particularly regarding potential overlap with TSCs. By leveraging this knowledge, researchers can enhance cell isolation techniques, advancing the clinical application of ES cells.

**Keywords:** Embryonic Stem Cells (ESCs); Stem Cell Markers; Pluripotency Maintenance; Cell Isolation Techniques; Tumor Stem Cells (TSCs)

**1. Introduction**

Embryonic stem cells (ESCs) represent a group of pluripotent stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo [1]. These remarkable cells possess the unique ability to maintain both pluripotency and self-renewal, a property essential for their potential clinical applications [1] **(Fig 1).**



**Figure 1:** Depiction of the different properties of Embryonic stem cells A) Undifferentiated cells C) Pluripotency C) Self –renewal property.

Pluripotency refers to the capability of ESCs to differentiate into all cell lineages found in living organisms while retaining an undifferentiated state during in vitro culture. This exceptional attribute makes ESCs highly desirable candidates for various therapeutic and regenerative medicine approaches [1].

However, one of the key challenges in the clinical use of ESCs lies in their reliable identification and distinction from other cell types, particularly tumor cells, to avoid potential risks and complications [1]. In this regard, understanding the specific gene expression patterns and identifying distinctive molecule markers associated with ESCs becomes imperative. These markers serve as crucial tools for the identification, isolation, and subsequent study of ESCs.

Researchers have made significant progress in identifying numerous cell surface markers and generic molecular markers that can serve as indicators of undifferentiated ESCs, particularly in the context of the human species [1]. Additionally, proteins involved in various signaling pathways have been recognized for their influential roles in determining cell fate. Interestingly, certain lectins and peptide analogs have demonstrated specific binding affinity to ESCs, further augmenting the potential repertoire of identification tools [1]. However, it is essential to acknowledge the existence of an obstacle that arises due to the overlap of ESC markers with those found in tumor stem cells. Consequently, caution must be exercised when utilizing these markers for ESC identification and isolation [1].

Furthermore, comprehending the mechanisms governing the pluripotency of human ESCs (hESCs) has emerged as a significant challenge in recent years. It has come to light that despite sharing similar embryonic origins, human and mouse ESCs exhibit differences in these regulatory mechanisms [2]. Therefore, delving deeper into the knowledge of these molecular markers is of utmost importance, not only for the proper utilization of ESCs but also for the elucidation of the intricate processes that control their pluripotency and self-renewal capacities [1]. A comprehensive understanding of ESC markers and their role in maintaining pluripotency and self-renewal is instrumental in advancing the field of regenerative medicine and developmental biology. In a study conducted by Smith and colleagues (2020), a novel surface marker, SALL4, was identified as a reliable indicator of undifferentiated hESCs [3]. This marker, when used in conjunction with other established markers like OCT4 and NANOG, facilitated the accurate identification and isolation of hESCs, overcoming the challenge posed by tumor stem cell marker overlap [3].

Additionally, several studies have investigated the significance of specific signaling pathways in regulating the pluripotency and self-renewal of ESCs. For instance, the Wnt signaling pathway has been identified as a crucial player in determining the fate of ESCs [4]. Upon activation, this pathway triggers a series of intracellular events that culminate in the maintenance of ESCs' undifferentiated state. On the other hand, inhibition of the Wnt pathway promotes ESC differentiation, emphasizing its significance in governing cell fate decisions [4]. Another prominent signaling pathway implicated in ESC pluripotency is the Hedgehog (Hh) pathway. Research by Li et al. (2019) revealed that the Hh pathway plays an essential role in maintaining the pluripotency of ESCs through the regulation of GLI transcription factors [5]. These findings provide valuable insights into the intricate network of signaling pathways that dictate the fate of ESCs, paving the way for potential therapeutic interventions. In addition to surface markers and signaling pathways, other molecules have also shown promise in serving as indicators of undifferentiated ESCs. For instance, small non-coding RNAs, such as microRNAs (miRNAs), have been demonstrated to have a significant impact on ESC pluripotency and differentiation [6]. These small RNA molecules regulate gene expression by targeting specific messenger RNAs (mRNAs), thereby influencing key cellular processes. Some miRNAs, such as miR-145, have been identified as negative regulators of ESC pluripotency, promoting their differentiation towards specific lineages [6]. In contrast, miR-302 and miR-367 have been recognized for their role in maintaining ESC pluripotency and inhibiting differentiation [6]. These findings emphasize the intricate regulatory network governing ESC behavior and provide valuable insights for potential therapeutic applications.

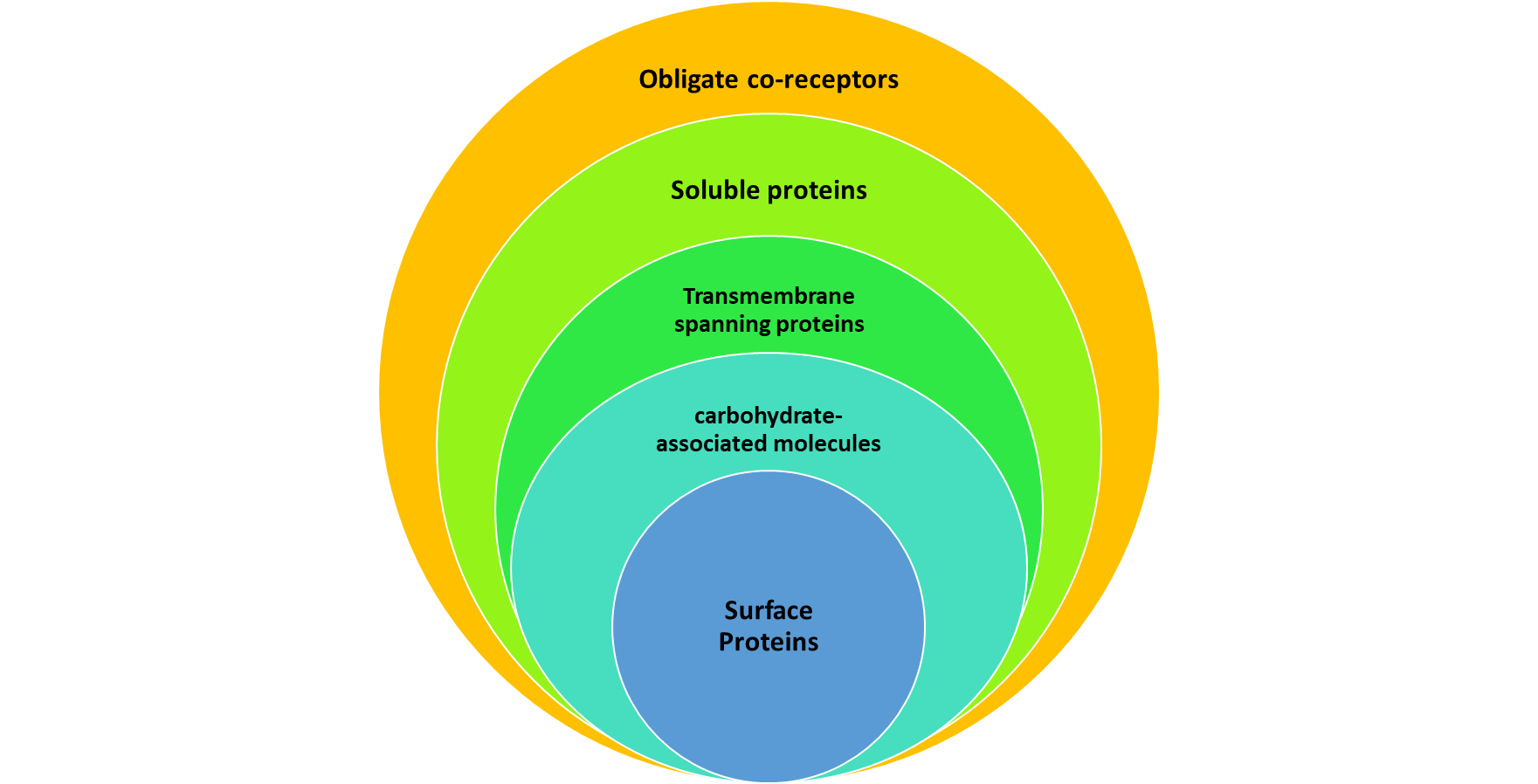
While these advancements contribute significantly to our understanding of ESC markers and their regulatory mechanisms, challenges persist in discerning the differences between human and mouse ESCs. Studies have revealed distinct gene expression patterns and epigenetic modifications in human and mouse ESCs, highlighting the need for further investigation [2]. For instance, a study by Chen et al. (2018) demonstrated that human and mouse ESCs exhibit differences in the regulation of X-chromosome inactivation, a crucial process that maintains pluripotency [7]. Such disparities necessitate the careful consideration of species-specific differences when applying findings from mouse ESC studies to human ESCs.

Embryonic stem cells (ESCs) represent a promising avenue for various therapeutic and regenerative medicine approaches due to their pluripotency and self-renewal capabilities. The identification and characterization of specific molecule markers associated with undifferentiated ESCs are critical for their successful clinical application. Researchers have made considerable progress in identifying cell surface markers, generic molecular markers, and signaling pathways that influence ESC pluripotency and self-renewal. Nevertheless, caution is warranted to distinguish ESC markers from those of tumor stem cells. Additionally, understanding the differences between human and mouse ESCs remains a significant challenge, necessitating further investigation into species-specific regulatory mechanisms.

Future research efforts should focus on expanding our understanding of ESC markers and their functions to facilitate safer and more effective clinical applications. By harnessing the potential of ESCs and unlocking the mechanisms governing their pluripotency and self-renewal, we can pave the way for groundbreaking advancements in regenerative medicine and developmental biology. This, in turn, holds the promise of transforming the landscape of modern medicine and improving the lives of countless individuals.

**2. Cell Surface Markers**

Cell surface proteins play a crucial role in recognizing and differentiating cell types due to their selective binding with signal molecules. These specialized membrane proteins can act as markers, aiding in the identification of specific cell types. However, it is important to note that certain membrane markers overlapping with tumor cell types pose challenges in distinguishing embryonic stem cells (ESCs) without compromising the integrity of the cell membrane [1] **(Fig 2).**



**Figure 2:** Depicting the nature of different embryonic stem cell markers .Some markers are surface proteins ,Some are carbohydrate based molecules ,while others are transmembrane based proteins , Soluble type of proteins and others are obligate co-receptors.

***2.1. Stage Specific Embryonic Antigens (SSEA)***

SSEA markers, characterized by three monoclonal antibodies recognizing specific carbohydrate epitopes associated with lacto- and globo-series glycolipids, namely SSEA-1, SSEA-3, and SSEA-4, play essential roles in controlling cell surface interactions during developmental processes [3]. SSEA-1 (CD15/Lewis x) is notably expressed on the surface of murine embryos at the pre-implantation stage, as well as in mouse and human germ cells, and teratocarcinoma stem cells. However, it is absent in human ESC and human embryonic carcinoma cells [4]. Beyond embryonic stages, SSEA-1 expression has been observed in various tissues in adults, such as the oviduct epithelium, endometrium, epididymis, as well as specific regions of the brain and kidney tubules [5]. Interestingly, SSEA-1 expression increases during differentiation in human cells, while it decreases during differentiation in mouse cells.

On the other hand, SSEA-3 and SSEA-4 are synthesized during oogenesis and are found in the membranes of oocytes, zygotes, and early cleavage-stage embryos [6]. Both SSEA-3 and SSEA-4 are expressed in undifferentiated primate ESCs. While SSEA-4 is absent in murine ESCs, it becomes detectable following differentiation, notably in human embryonic germ (EG) cells, human teratocarcinoma stem cells, and ESCs [7]. The presence and absence patterns of these SSEA markers across different stages of development and in various cell types highlight their significance as valuable tools for identifying and characterizing specific cell populations during embryonic and tissue development.

***2.2. Cluster of Differentiation (CD) Antigens***

CD antigens, a group of surface proteins belonging to various classes such as integrins, adhesion molecules, glycoproteins, and receptors, serve as valuable markers for identifying and characterizing different cell populations [8]. In particular, several CD antigens have been associated with mouse and human embryonic stem cells (ESCs). Pluripotent human ESCs express CD9, CD24, CD133, CD90, and CD117, while CD133 also serves as a marker for hematopoietic stem cells [9-11].

Integrins, a subclass of CD antigens, are α/β heterodimeric cell surface receptors responsible for mediating cell attachment to surrounding tissues [12]. These receptors play essential roles in cell adhesion, signaling, migration, growth, and survival [12]. Integrins collaborate with other proteins, such as cadherins, immunoglobulin superfamily cell adhesion molecules, selectins, and syndecans, to facilitate cell-cell and cell-matrix interactions and communication [13]. Binding to various components of the extracellular matrix (ECM), including fibronectin, vitronectin, collagen, and laminin, integrins conduct both outside-in and inside-out signaling. The outside-in signaling conveys information from the ECM to the cell, while the inside-out signaling can activate other integrins, facilitating rapid and flexible responses to changes in the cellular environment [14].

The integrin family consists of multiple α and β subunits that combine to form diverse integrin types, each with distinct tissue distributions and overlapping ligand specificities [16]. Within this family, integrins α5β1, αvβ5, α6β1, and α9β1 have been found to play pivotal roles in maintaining stemness in undifferentiated mouse ESCs [17]. Specifically, integrin α6 (CD49f/CD29), a 120-kDa protein with two splice variants (α6A and α6B), functions as a receptor for laminins and mediates cellular adhesion events on the basal membrane [18]. In hematopoietic stem and progenitor cells, integrin α6 (CD49f/CD29) has been demonstrated to play a critical role in homing to the bone marrow [19], while in human prostate carcinoma cells, it exhibits significance in cell behavior [20].

The presence and functional roles of these integrins in ESCs emphasize their importance in constructing and maintaining ESC niches [15]. Understanding the intricacies of integrin-mediated signaling pathways and their interactions with the ECM and other cell surface molecules will contribute to advancing our knowledge of stem cell behavior and provide valuable insights for regenerative medicine and tissue engineering applications. These integrins may serve as potential targets for controlling ESC behavior, differentiation, and fate determination, paving the way for future therapeutic interventions and advancements in regenerative medicine.

***2.3. TRA-1-60 and TRA-1-81***

TRA-1-60 and TRA-1-81 antigens, present on the surfaces of human embryonal carcinoma (EC) cells and human pluripotent stem cells, serve as valuable markers for identifying and isolating ESCs [21]. These antigens are also expressed in teratocarcinoma and embryonic germ cells [22]. While the TRA-1-60 antibody recognizes a neuraminidase-sensitive epitope of a proteoglycan, the TRA-1-81 antibody binds to a neuraminidase-insensitive epitope of the same molecule, which has been suggested to be a variant of the protein podocalyxin [23]. However, it is essential to note that TRA-1-60 is also detectable in the serum of patients with germ cell tumors, posing a challenge in its use as a specific marker for ESCs [24].

***2.4. Frizzled (Fzd)***

Fzd, a member of the seven-transmembrane-spanning G-protein-coupled receptor (GPCR) superfamily, plays a critical role in the transduction of Wnt signals [25]. Its large extracellular N-terminal region contains a cysteine-rich domain (CRD), facilitating binding to Wnt proteins [26]. Wnt signals are transmitted through the family of Fzd receptors, and upon Wnt protein binding, Fzd forms a complex with co-receptors LRP5 or LRP6 to activate the canonical Wnt/β-catenin pathway by inhibiting β-catenin phosphorylation by GSK3-β. Additionally, certain Wnt proteins can activate the Fzd/Ca2+ and Fzd/PCP (planar cell polarity) pathways, further diversifying the signal transduction mechanisms [27].

The intracellular C-terminus of Fzd binds to the PDZ domain of Dvl proteins, an essential downstream signaling component, linking Fzd to various intracellular signaling pathways [27]. The mammalian Fzd subfamily encompasses ten members (Fzd1 to Fzd10), and their expression in both mouse and human embryonic stem cells (ESCs) highlights their significance in mediating diverse signaling pathways [28].

Moreover, some Fzd receptors can interact with other secreted proteins, such as Norrin and R-Spondin, adding complexity to their functional roles [27]. These interactions contribute to the fine-tuning of Wnt signaling, thereby influencing cell fate decisions and developmental processes during embryogenesis. The diverse repertoire of Fzd receptors and their involvement in various pathways underscores their pivotal position in orchestrating cellular responses to Wnt signaling cues. A comprehensive understanding of the intricacies of Fzd signaling in ESCs may hold the key to unlocking their full potential in regenerative medicine and tissue engineering applications. Further research into the specific roles and regulatory mechanisms of individual Fzd receptors will deepen our knowledge of Wnt signaling in ESCs and pave the way for innovative therapeutic strategies.

***2.5. Stem Cell Factor (SCF or c-Kit Ligand)***

Stem Cell Factor (SCF), also known as kit-ligand, KL, or steel factor, is a cytokine that interacts with the c-Kit receptor (CD117) [29]. SCF can exist in two forms: a transmembrane protein and a soluble protein. The soluble SCF exists as a non-covalently associated homodimer, characterized by glycosylation and significant secondary structure, including regions of alpha helices and beta sheets. Within each SCF monomer, two intra-chain disulfide bridges are present, and the N-terminal 141 residues have been identified as a functional core (SCF1-141). This region includes the dimer interface and portions that bind and activate the receptor Kit [29].

SCF transmits signals through ligand-mediated dimerization of its receptor, Kit, which is a type III receptor protein-tyrosine kinase related to other receptors, such as those for platelet-derived growth factor (PDGF), macrophage colony-stimulating factor, Flt-3 ligand, and vascular endothelial growth factor (VEGF). Upon binding to Kit, SCF induces receptor dimerization and activates protein kinase activity [29]. SCF is expressed in various fibroblast-type cells and sites where hematopoiesis occurs, such as the fetal liver and bone marrow. The cytokine plays a pivotal role in hematopoiesis, spermatogenesis, and melanogenesis. Notably, the survival of differentiating embryonic stem cells is dependent on the SCF-KIT pathway, highlighting its significance in regulating stem cell fate [30].

Given the essential roles of SCF in hematopoiesis and stem cell maintenance, understanding its regulatory mechanisms and interactions with the c-Kit receptor holds tremendous promise for advancing regenerative medicine and therapeutic interventions. Further research into the SCF-KIT pathway and its impact on stem cell behavior will contribute to the development of novel strategies to harness the potential of stem cells for tissue repair and regeneration. Moreover, exploring the intricate network of signaling pathways involving SCF and its receptor will deepen our understanding of cell fate determination and differentiation processes, opening new avenues for innovative therapeutic approaches in various fields of medicine.

***2.6. Cripto (TDGF-1)***

The Cripto gene, also known as teratocarcinoma-derived growth factor-1 (TDGF-1), encodes a novel human growth factor structurally related to epidermal growth factor. In embryonic development, Cripto serves as an obligate co-receptor for transforming growth factor β (TGF-β) ligands, including nodals, growth and differentiation factor 1 (GDF1), and GDF3. Apart from its crucial role during embryogenesis, Cripto functions as an oncogene, exhibiting high expression levels in tumors and promoting tumorigenesis through mechanisms such as activation of mitogenic signaling pathways and antagonism of activin signaling [31].

**3. Transcription Factors**

Nuclear genes play pivotal roles in vital cellular functions, with transcription factors serving as critical regulators of gene expression. Under normal conditions, some transcription factors remain inactive until specific signal transduction events trigger their binding to cognate recognition sequences. The emergence and function of unique genes within the nucleus indicate the cell's response to specific conditions. Hence, monitoring the expression of these genes can serve as valuable markers for specific cellular states. **Table 1** lists the transcription factors expressed in embryonic stem cells (ESCs).

**Table 1:** Nuclear transcription factors and their characteristics.

|  |  |  |
| --- | --- | --- |
| Nuclear transcription factors | Characteristics | |
| Oct-3/4 | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells | |
| Sox2 | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells, neural stem (NS) cells | |
| KLF4 | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells | |
| Nanog | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells | |
| Markers |  | |
| Rex1 (Zfp42) | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells | |
| UTF1 | Mouse human ES cells, germ line tissues in mouse and human, embryonal carcinoma (EC) cells | |
| ZFX | Murine ES cells, human ES cells, hematopoietic stem cells, embryonal carcinoma (EC) cells | |
| TBN | Mouse, human inner cell mass | |
| FoxD3 | Murine ES cells, human ES cells, embryonal carcinoma (EC) cells | |
| HMGA2 | Mouse ES cells, human ES cells | |
| NAC1 | human ES cells and mouse ES cells | |
| GCNF (NR6A1) | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells | |
| Stat3 | Murine ES cells, Human ES cells, embryonal carcinoma (EC) cells | |
| LEF1, TCF3 | Mouse ES cells, Human ES cells, embryonal carcinoma (EC) cells | |
| Sall4 | Murine ES cells, Human ES cells, embryonal carcinoma (EC) cells | |
| Fbxo15 | Mouse ES cells, early embryos, and testis tissue, embryonal carcinoma (EC) cells | |
| ECAT genes | | |
| ECAT11 (FLJ10884/ L1TD1) | Human ES cells, embryonal carcinoma (EC) cells | |
| Ecat1 | Mouse oocytes, embryonal carcinoma (EC) cells | |
| ECAT9 (Gdf3) | Human ES cells, embryonal carcinoma (EC) cells | |
| Dppa genes | |  |
| Dppa5 (ESG1) | Mouse ES cells, Human ES cells, embryonal carcinoma (EC) cells | |
| Dppa4 | Mouse ES cells, Human ES cells, embryonal carcinoma (EC) cells | |
| Dppa2 (ECSA) | Mouse ES cells, Human ES cells, embryonal carcinoma (EC) cells | |
| Dppa3 (Stella) | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells, primordial germ cells, oocytes, preimplantation embryos | |

***3.1. CORE Nuclear Transcription Factors***

In 2006, Yamanaka and colleagues demonstrated the generation of pluripotent stem cells from mouse embryonic fibroblasts through the combined expression of four factors: Oct4, c-Myc, Sox2, and Klf4 [32]. Following this breakthrough, induced pluripotent stem (iPS) cells have been successfully derived from various somatic cells by over-expressing a defined set of genes. However, concerns arose due to the potential tumorigenicity associated with c-Myc reactivation, as approximately 20% of iPS cell offspring developed tumors [33]. In response, researchers developed a modified protocol for iPS cell generation that eliminates the need for the Myc retrovirus, resulting in significantly fewer non-iPS background cells and consistently high-quality iPS cells [34].

Further investigations have suggested that the number of reprogramming factors can be reduced in cases where somatic cells express sufficient levels of endogenous complementing factors. For instance, adult mouse neural stem cells (NSCs) were found to express higher levels of endogenous Sox2 and c-Myc compared to embryonic stem cells (ESCs). Consequently, the introduction of Oct4, along with either Klf4 or c-Myc, was sufficient to generate iPS cells from NSCs [35].

In recent advancements, it was discovered that the expression of the transcription factor Oct4 alone is adequate to directly reprogram adult mouse NSCs to pluripotency. This finding highlights Oct4 as both required and sufficient for the direct reprogramming of NS cells into pluripotent stem cells [36]. Similar results were obtained using human neural stem cells (NSCs) [37].

These breakthroughs in iPS cell generation have profound implications for regenerative medicine and disease modeling. By understanding the key factors involved in reprogramming somatic cells into pluripotent stem cells, researchers can explore new avenues for potential therapies and gain valuable insights into developmental biology and disease pathogenesis. Furthermore, the continuous refinement of reprogramming techniques ensures safer and more reliable generation of iPS cells for future clinical applications.

***3.1.1. Octamer-binding Protein 4 (Oct4)***

Oct4, also known as Oct3/4 or POU5F1, belongs to the POU family of transcription factors and plays a crucial role in regulating stem cell pluripotency and differentiation [38]. The POU domain within these transcription factors is essential for their functions, while regions outside the POU domain exhibit limited sequence conservation and are not critical for DNA binding [39]. Oct4 ortholog genes are structurally organized and highly conserved across different mammalian species, such as humans, bovines, mice, and rats [40].

During early embryonic development, Oct4 expression is primarily restricted to pluripotent and germ line cells, and it is maintained in the inner cell mass (ICM) of blastocysts [41]. Differential expression of Oct4 is observed as the ICM differentiates into epiblasts (primitive ectoderm and embryonic ectoderm) and hypoblasts (primitive endoderm and embryonic endoderm) at 4.5 days post-coitum (dpc). While Oct4 expression is retained in the epiblast, as hypoblast cells differentiate into visceral and parietal endoderms, Oct4 protein levels transiently increase before decreasing to undetectable levels. During gastrulation at 7.5 dpc, Oct4 expression is progressively repressed in the epiblast [41].

Oct4 expression is also maintained in pluripotent cell lines derived from the ICM, epiblasts, and primordial germ cells (PGCs), such as embryonic stem cells (ESCs), embryonic carcinoma (EC) cells, and embryonic germ (EG) cells, respectively, as long as they remain undifferentiated [41].

Furthermore, Oct4 plays a pivotal role in regulating the expression of several genes during early development, including Sox2, Fgf4, Rex1, hCG, and Utf1, contributing to the maintenance of pluripotency and the proper differentiation of cells [38].

Understanding the regulatory functions of Oct4 in stem cell biology and early embryonic development is critical for advancing regenerative medicine, disease modeling, and reproductive technologies. Insights into Oct4-mediated gene regulation and its interactions with other key transcription factors will aid in manipulating stem cell fate and developing novel therapeutic strategies for various medical applications. Continued research into the complex molecular networks involving Oct4 promises to unravel new discoveries with profound implications for both basic science and clinical medicine.

***3.1.2. Sry-related High-mobility Group (HMG) Box-containing (Sox) Family***

Sox2, a member of the Sox gene family, belongs to the HMG box transcription factors that functionally interact with POU domain proteins [42]. Similar to Oct3/4, the Sox gene family is involved in maintaining pluripotency, but Sox2 is specifically associated with multipotent and unipotent stem cells, while Oct3/4 is exclusively expressed in pluripotent stem cells. Notably, Sox2 was one of the first genes used for inducing iPS cells by various research groups [43].

The Sox2 gene contains at least two regulatory regions that are specifically active in pluripotent embryonic cells. Its expression pattern is similar to Oct4, observed in human and mouse pre-implantation embryos, as well as in mES, hES, mEC, and hEC cells. During later development, Sox2 is co-expressed with Oct4 in post-migratory primordial germ cells [44]. Furthermore, early mouse embryos exhibit co-expression of Oct4, Sox2, and osteopontin in the same cells. Although Sox1 demonstrates a similar efficiency to Sox2 in producing iPS cells, other genes in the Sox family, such as Sox3, Sox15, and Sox18, also play a role in generating iPS cells, albeit with reduced efficiency [42].

Understanding the distinctive functions of Sox2 in comparison to Oct4 and other Sox genes is essential for comprehending the intricacies of pluripotency regulation and stem cell differentiation. The identification of key regulatory elements and interactions with other transcription factors will offer valuable insights into the molecular mechanisms governing cell fate determination and provide novel opportunities for harnessing stem cells for regenerative medicine and therapeutic applications. Ongoing research in this field holds great promise for advancing our understanding of stem cell biology and revolutionizing medical interventions for various diseases and disorders.

***3.1.3. Krupple-like Factor (Klf) Family***

The Krüppel-like factor (Klf) family of transcription factors plays a crucial role in regulating various biological processes, including cell proliferation, differentiation, development, and apoptosis. Members of the Klf family are characterized by three Cys2 His2 zinc fingers located at the C-terminus, separated by a highly conserved H/C link. These factors demonstrate similar affinities for different GC-rich DNA binding sites and can compete with each other for occupancy of these sites. Moreover, Klf proteins share a significant degree of homology with the specificity protein (Sp) family of zinc-finger transcription factors, leading to their binding similarity in numerous genes.

Klf5, also known as intestine-enriched Krüppel-like factor or Bteb2, is a pioneer member of this family with critical developmental functions. It has been reported that Klf5 directly regulates the transcription of Oct3/4 and Nanog, which are essential for embryonic stem cells (ESCs) renewal and pluripotency maintenance [45]. On the other hand, Klf4 and Klf2 are functionally redundant in controlling the self-renewal and pluripotency of ESCs. They also regulate the expression of other pluripotency-related transcription factors in ESCs, including Nanog, Tcl1, Esrrb, Sall4, Tcf3, Mycn, and Fbxo15. However, individual Klfs were found to be dispensable for the self-renewal of ESCs, suggesting that they may converge and work together to regulate common targets [46]. In ESCs, these Klfs extensively colocalize to specific genomic regions, further supporting their cooperative roles in regulating pluripotency-related genes [47].

The intricate interplay of Klf family members in the maintenance of pluripotency and the regulation of key transcription factors highlights their significance in stem cell biology. Understanding the complex interactions and target regulation of Klfs contributes to elucidating the mechanisms underlying ESC self-renewal and differentiation processes. These insights hold great potential for advancing regenerative medicine and therapeutic strategies by manipulating pluripotency and stem cell fate for various clinical applications. Continued investigation into the Klf family and its interconnectivity with other regulatory factors will enhance our comprehension of stem cell biology and drive innovations in medical research and treatments.

***3.1.4. Nanog***

Nanog, a critical transcription factor, plays a central role in maintaining pluripotency and self-renewal in both mouse and human embryonic stem cells (ESCs) [48]. Chambers et al. [49] demonstrated that Nanog holds a central position in the transcription factor hierarchy that defines ESC identity. Nanog mRNA is present in pluripotent ES and embryonic germ (EG) cells, as well as in mouse and human embryonic carcinoma (EC) cells. However, its expression is downregulated early during ESC differentiation, consistent with its intimate association with pluripotent stem cell identity. The restricted expression of Nanog aligns with the transient potential for ESC generation observed in the inner cell mass (ICM) during early embryonic stages, which is lost during implantation [49]. Mitsui [50] further confirmed the significance of Nanog in maintaining pluripotency in mouse epiblasts and ESCs. Nanog's role in promoting ESC self-renewal is independent of the LIF/Stat3 pathway. Nanog-deficient ICMs failed to generate epiblasts and only gave rise to parietal endoderm-like cells. In the absence of Nanog, ESCs lost pluripotency and differentiated into extraembryonic endoderm lineage, underscoring Nanog's crucial role in defining ESC identity [51].

Notably, Nanog mRNA is detected in pluripotent mouse and human cell lines but is absent in differentiated cells. During preimplantation embryo development, Nanog expression is restricted to founder cells, from which ESCs can be derived. Endogenous Nanog acts in parallel with cytokine stimulation of Stat3 to induce ESC self-renewal. Elevated Nanog expression from transgene constructs alone is sufficient to drive the clonal expansion of ESCs, bypassing the need for Stat3 activation while maintaining Oct4 levels. The dependence on cytokines, multilineage differentiation potential, and embryo colonization capacity are fully restored upon transgene excision, emphasizing the crucial role of Nanog in orchestrating ESC pluripotency and fate determination [51].

The pivotal function of Nanog in maintaining ESC identity and self-renewal is of great interest in stem cell biology and regenerative medicine. Understanding the molecular mechanisms and regulatory networks involving Nanog offers promising avenues for controlling pluripotency and enhancing the efficiency of reprogramming somatic cells into induced pluripotent stem cells (iPSCs). Insights into Nanog-mediated signaling pathways and its interplay with other key transcription factors are valuable for harnessing the potential of ESCs and iPSCs for various clinical applications, including tissue engineering, disease modeling, and personalized therapies. Continued research into Nanog and its regulatory functions will undoubtedly contribute to advancing our knowledge of stem cell biology and translating it into transformative medical interventions.

***3.2. Reduced Expression 1 (Rex1 or Zfp-42)***

The Rex1 (Zfp42) gene encodes a zinc finger family transcription factor highly expressed in both mouse and human embryonic stem cells (ESCs). The Rex1 protein contains four nucleic acid zinc finger motifs and an acidic domain [52]. Originally identified in F9 embryonal carcinoma (EC) cells, Rex1 is down-regulated upon retinoic acid (RA) treatment to induce differentiation. It shares similarity with Yy1, an evolutionarily conserved component of the polycomb-related complex [53]. While Rex1's detailed function remains unclear, it is considered a marker of pluripotency in various stem cells, including multipotent adult progenitor cells and amniotic fluid cells [54]. Studies using conventional gene targeting strategies demonstrated that Rex1 is dispensable for both ESC pluripotency maintenance and embryonic development [55]. Thus, Rex1 is primarily regarded as a marker of pluripotency without functional significance, akin to alkaline phosphatase activity. Rex1's role in ESC differentiation has been investigated through the generation of Rex1 double knockout ESC lines, which showed increased expression of ectoderm, mesoderm, and endoderm markers compared to wild-type cells, suggesting that Rex1 limits retinoic acid-induced differentiation in ESCs [56].

***3.3. Undifferentiated Embryonic Cell Transcription Factor (UTF1)***

UTF1, a transcriptional co-activator, interacts with the metal-binding motif of activation transcription factor-2 (ATF-2) and plays a vital role in initiating ESC differentiation. Knockdown of UTF1 in ES and carcinoma cells results in substantial delay or blockage of differentiation [57]. It is primarily expressed in pluripotent ESCs, where it tightly associates with chromatin in both mouse and human ESCs, possibly contributing to maintaining the necessary epigenetic environment for pluripotency [58]. UTF1's gene contains a regulatory element that selectively interacts with an Oct3/4 and Sox-2 complex, and Oct4 and Sox2 have been shown to regulate UTF1 expression [60]. Co-expression of UTF1 with reprogramming factors c-Myc, Oct4, Sox2, and KLF4, along with siRNA knockdown of p53, significantly enhances the efficiency of induced pluripotent stem cell (iPSC) generation [61]. In mouse embryos, UTF1 mRNA is present in the inner cell mass, primitive ectoderm, and extra-embryonic tissues [62]. Its expression is primarily restricted to pluripotent cells (ICM cells) in mouse blastocysts, and its levels rapidly decrease upon differentiation [63].

***3.4. X-linked Zinc Finger Protein (ZFX)***

The ZFX gene is expressed from the inactive X chromosome and is structurally similar to its homologue on the Y chromosome, ZFY [64]. ZFX and ZFY transcripts encode proteins with highly acidic amino-terminal domains and carboxy-terminal zinc-finger motifs associated with nucleic acid-binding. Both ZFY and ZFX may function as possible transcriptional activators involved in sex determination. Multiple alternatively spliced transcript variants of ZFX, encoding different isoforms, have been identified and may have distinct functions [65]. Conditional gene targeting studies in mice have suggested that ZFX is required for the self-renewal of embryonic and hematopoietic stem cells [66]. Additionally, ZFX may be involved in B-cell proliferation and expansion and contribute to lymphocyte homeostasis [67].

***3.5. Taube Nuss (Tbn)***

Tbn, highly conserved between humans and mice, is the founding member of a novel class of proteins with essential developmental functions. It is restricted to inner cell mass (ICM) cells and is essential for the survival of ICM cells [68]. Tbn expression has also been detected in human embryonic stem cells [69]. In the absence of Tbn, ICM cells undergo apoptosis, leading to an imbalance between cell death and survival in early embryos, resulting in the demise of pluripotent ICM cells while trophectoderm cells survive.

***3.6. Forkhead Box D3 (FoxD3)***

FoxD3, a member of the Forkhead box family, features a winged-helix DNA-binding structure and plays a crucial role in embryonic development [70]. This transcriptional regulator is necessary for pluripotency maintenance during pre-implantation and peri-implantation stages of mouse embryonic development [71] and is also involved in trophoblast formation [72]. In the mammalian neural crest, FoxD3 is required for its maintenance; FoxD3 (-/-) mouse embryos fail around implantation, leading to a loss of neural crest-derived structures [73]. FoxD3 collaborates with Oct4 and Nanog to sustain ESC pluripotency [74].

***3.7. HMGA2***

HMGA2, an architectural transcription factor, lacks direct transcriptional activation capacity. Instead, it regulates gene expression by altering DNA conformation through binding to AT-rich regions and interacting directly with other transcription factors. HMGA2 is abundantly and ubiquitously expressed and plays a crucial role during embryonic development [75]. It promotes stem cell self-renewal, and its reduced expression is linked to stem cell aging [76]. HMGA2's expression is typically low in normal adult tissues but is associated with various cancers when overexpressed or rearranged [77].

***3.8. Nucleus Accumbens-1 (NAC1)***

NAC1, a nuclear factor belonging to the Pox virus and zinc finger/bric-a-brac tramtrack broad complex (POZ/BTB) domain family, was initially identified in a unique neuronal forebrain structure involved in reward motivation and addictive behaviors [78]. NAC1 recruits HDAC3 and HDAC4 to repress gene expression in neuronal cells, particularly co-repressing other POZ/BTB proteins in the central nervous system [79]. NAC1 is upregulated in several tumor types, including breast, renal cell, and hepatocellular carcinoma, as well as high-grade ovarian serous carcinoma, where it is implicated in chemoresistance [80]. In ESCs, NAC1 is part of an extended transcriptional network involving Oct4, Sox2, Nanog, Sall1, KLF4, and Sall4 [82].

***3.9. Germ Cell Nuclear Factor (GCNF)***

GCNF, also known as nuclear receptor subfamily 6 group A member (NR6A1), is an orphan member of the nuclear receptor gene superfamily [83]. It is expressed during nervous system development and specific stages of maturing germ cells in the adult ovary and testis. GCNF may be involved in gametogenesis, neurogenesis, and normal embryonic development during gastrulation [89]. In mice, inactivation of GCNF leads to abnormal posterior development, impaired midbrain development, insufficient neural tube closure, and eventual embryonic death [90]. GCNF acts as a repressor of Oct4 and protamine genes and plays a critical role in gene expression control during embryogenesis and spermatogenesis [91].

***3.10. Stat3***

Stat3, an essential signaling molecule for many cytokines and growth factor receptors, is crucial for murine fetal development [94]. In mouse ESCs, Stat3 is activated by binding LIF to the LIF receptor, leading to Stat3 translocation into the nucleus and activation of downstream genes, including Sall4, Myc, and KLF4 [95]. Suppression of Stat3 induces ESC differentiation [96], while constitutive activation maintains ESCs in the undifferentiated state, even in the absence of LIF [97]. Stat3 is constitutively activated in various human tumors [98] and possesses oncogenic potential [99] and anti-apoptotic activities [100]. Transcriptional activation is regulated by phosphorylation at Tyr705, inducing dimerization, nuclear translocation, and DNA binding [101]. Phosphorylation at Ser727 through the MAPK or mTOR pathways appears to modulate transcriptional activation [102]. The expression of Stat3 isoforms, Stat3α (86 kDa) and Stat3β (79 kDa), varies depending on the cell type, ligand exposure, or cell maturation stage [103].

***3.11. LEF1 and TCF***

LEF1 and TCF belong to the HMG DNA-binding protein family of transcription factors, which includes lymphoid enhancer factor 1 (LEF1), T-cell factor 1 (TCF1), TCF3, and TCF4 [104]. Originally identified as regulators of early lymphoid development [105], LEF1 and TCF1 act downstream in Wnt signaling. They bind to Wnt response elements, providing docking sites for β-catenin, which translocates to the nucleus upon Wnt signaling activation to promote the transcription of target genes. LEF1 and TCF proteins exhibit dynamic expression during development and aberrant activation of the Wnt signaling pathway in many types of cancers, including colon cancer [106]. TCF3 (also known as TCF7L1) plays a crucial role in integrating Wnt signaling with stem cell differentiation regulation [107].

***3.12. SALL Family***

The SALL gene family (also known as Hsal) plays important roles in regulating developmental processes in various organisms. It comprises SALL1, SALL2, SALL3, and SALL4, initially cloned from a DNA sequence homologous to the Drosophila gene sal [108]. SALL4 is an essential regulator of Oct4 and is required for ESC pluripotency [109]. Downregulation of Sall4 in mouse ESCs results in their respecification to the trophoblast lineage when grown in feeder-free conditions. While Sall4 is essential for ESC stabilization, it is not required for pluripotency maintenance [110]. SALL4 and Oct4 balance the expression of other SALL gene family members, particularly Sall1 and Sall3, which are expressed in both murine and human ESCs. Deletion of Sall1 and Sall3 in mice leads to perinatal death due to developmental defects [111].

***3.13. F-box 15 (FBXO15)***

FBXO15, a member of the F-box protein family characterized by a 40-amino acid F-box motif, is a novel target of Oct3/4. However, it is dispensable for ESC self-renewal, development, and fertility [112]. FBXO15 is predominantly expressed in undifferentiated mouse ESCs, and its expression rapidly disappears upon Oct3/4 inactivation. Its expression profile is nearly identical to that of Oct3/4 and is primarily restricted to ESCs, early embryos, and testicular tissue.

***3.14. ESC Associated Transcript (ECAT) Genes***

The ECAT genes play essential roles in stem cell biology. ECAT1 encodes a K homology RNA-binding (KH) domain-containing RNA-binding protein specifically expressed in mouse oocytes [113]. ECAT4 was identified as Nanog, a master regulator of mESC and hESC maintenance [114]. ECAT5 was identified as embryonic stem cell-expressed Ras (ERas), a Ras-like oncogene that regulates mESC proliferation [115]. ECAT9 was identified as growth and differentiation factor 3 (GDF3), an important factor that helps maintain mESC pluripotency by inhibiting bone morphogenetic protein (BMP) signaling [116]. ECAT11, also known as FLJ10884 or L1TD1, is abundantly expressed in undifferentiated hESC. Studies have shown that L1TD1 is a downstream target of Nanog and serves as a useful marker in identifying undifferentiated human ESC [117].

***3.15. Developmental Pluripotency-associated (DPPA) Genes***

The DPPA molecules, a group of five proteins related by name only, are described as a set of Oct4-related genes and serve as markers for early embryonic and germline pluripotent cells. DPPA5, also known as ESG1, is a KH domain-containing protein expressed in EG cells and ESCs, making it a potential marker for ESCs [118]. DPPA3, also called Stella, is expressed in primordial germ cells, oocytes, preimplantation embryos, and pluripotent cells [119]. It serves as a marker of pluripotency and plays roles in transcriptional repression, cell division, and maintenance of pluripotency in mice and humans. Related intron-less loci are expressed in germ cell tumors [120]. DPPA4 is reported to be a nuclear factor associated with active chromatin, regulating ESC differentiation into a primitive ectoderm lineage [121].

**Signal Pathway-related Intracellular Markers**

Several intracellular signal pathways play critical roles in maintaining ESC self-renewal and pluripotency, making them important markers of ESC fate. The core signal pathways regulating ESC self-renewal and pluripotency include LIF-STAT3, BMP-SMAD, TGF-β/Activin/Nodal, IGF-IR, FGFR, and Wnt-β-catenin [122]. LIF-STAT3 and BMP-SMAD are particularly critical for mouse ESC self-renewal [123], though LIF-STAT3 is not active in undifferentiated hESC [124]. While the BMP signaling pathway plays a significant role in both mouse and human ESCs [125], its upstream effectors and effects often differ. For example, BMP4 maintains pluripotency in mESCs but induces trophectoderm differentiation in hESCs [126, 127]. SMAD proteins transduce BMP signals by regulating downstream gene expression through interactions with other DNA-binding proteins in the nucleus, and SMAD1/5/8 may serve as markers for ESCs due to their high expression levels [128].

Wnt and TGF-β/Activin/Nodal pathways are also crucial for self-renewal in both mouse and human ESCs. Wnt/β-catenin, a key regulator involved in cell growth and embryonic development [129], is highly expressed in ESCs and regulates their pluripotency [130]. Thus, it can be considered a marker for ESCs. TGF-β family members play a role in hESC cell fate decisions [131]. Activin/Nodal signaling via Smad2/3 and Smad4 activation is necessary for maintaining hESC pluripotency and upregulating the transcription of Oct4 and Nanog [132]. Hence, Smad2/3 and Smad4 may also serve as markers in hESC. **Table 2** summarizes the potential markers among these pathways.

**Table 2:** Different markers, Characteristics and classification of Embryonic stem cells

|  |  |  |
| --- | --- | --- |
| Markers | Characteristics | Classification |
| SMAD1/5/8 | Mouse ES cells, embryonal carcinoma (EC) cells | Smad proteins ((R-Smad), BMP signalling pathway |
| SMAD4 | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells, early embryos, and testis tissue | Smad proteins (Co-SMAD), TGF- β /Activin/Nodal signalling pathway, BMP signalling pathway |
| SMAD2/3 | Human ES cells, embryonal carcinoma (EC) cells | Smad proteins ((R-Smad), TGF- β /Activin/Nodal signaling pathway |
| β-catenin | Mouse ES cells, human ES cells, embryonal carcinoma (EC) cells | Transcription activators, Wnt/β-catenin signaling pathway |

**Enzymatic Markers**

Both mouse and human ESCs exhibit elevated levels of alkaline phosphatase and telomerase. Alkaline phosphatase is prominently expressed on the cell membrane of ESCs, and in humans, TRA-2-49 and TRA-2-54 antibodies can detect alkaline phosphatase. In murine cells, detection is typically achieved through an enzymatic-based reaction [133]. Consequently, alkaline phosphatase staining serves as a reliable method to detect and assess pluripotency in ESCs. The NIH stem cell resource website provides a comprehensive list of some of these markers (<http://stemcells.nih.gov/info/scireport/appendixe.asp#eii>).

**Other Markers I**

In recent studies, researchers have explored the use of small molecules such as lectins or short peptides that specifically bind to ESC surface receptors. By labeling these molecules with quantum dots (QD) or fluorescence dyes, they can act as markers to label, identify, and isolate ESCs.

***6.1. Lectins***

Lectins are carbohydrate-binding proteins that recognize diverse sugar structures, making them valuable tools for identifying and characterizing cell surface glycosylation patterns [134]. Their application has contributed to the delineation of embryological developmental stages in some species and has facilitated the investigation and identification of specific cell types based on cell surface carbohydrate presentation [135]. In mouse ESCs, developmentally regulated glycans identified as lectin receptors are displayed on cell surfaces during preimplantation and implantation stages of development [136]. Lectins have also proven useful as markers for identifying mouse ESC-derived retinal progenitor cells for transplantation therapy [137] and for probing differentiated human ESCs [138]. Additionally, lectins have been employed as markers to define different stages of mouse embryogenesis and to characterize subpopulations in colonies of adherent hESCs.

***6.2. Peptides Specific for ES Cells***

Receptor-ligand interactions underlie numerous cellular biological processes, making the identification of ligands binding to specific cell targets fundamental for drug development, biomaterials, and diagnostic tools [139]. Phage display technology is a highly efficient method to discover novel biomarkers [140]. The technology involves fusing nucleotide sequences of random polypeptides with a phage coat protein, enabling display of chimeric proteins on the phage surface. By selecting for specific binding to the target of interest, a phage pool with increasing specific binding ability can be obtained. Ligands identified through phage display screens can bind to specific sites on target cells, serving as markers for recognizing and isolating these cells. Several small peptides specific to Rhesus Monkey Embryonic Stem Cells (R-ESCs) and mouse ESCs have been reported, with these peptides conjugated with quantum dots demonstrating the ability to target ESCs [141]. Peptides specific to human ESCs and human embryonal carcinoma cells (ECs) have also been identified [142]. When cultured on self-assembled monolayers presenting specific peptide sequences, ESCs expressed pluripotency markers at levels comparable to those cultured on Matrigel [142].

**Markers Overlapping with Tumor Stem Cells**

The unique characteristics of adult stem cells, including their longevity, self-renewal capacity, and multilineage differentiation potential, make them critical in normal physiological and pathological conditions [142]. When the differentiation potential of stem cells becomes impaired and their proliferative capacity becomes uncontrolled, these mutated, self-renewing stem cells may acquire tumorigenic properties, giving rise to cancer stem cells (CSCs) or tumor stem cells (TSCs) that play significant roles in carcinogenesis. CSCs have been isolated from various organs, including the breast, brain, blood (leukemia), skin (melanoma), head and neck, thyroid, cervix, and lungs [141]. Recent studies have utilized a range of CSC markers to distinguish tumor cells from normal tissues [142]. Interestingly, ESCs and CSCs share many common marker genes, which raises potential concerns regarding the use of ESC transplants.

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