



Stem Cells and Metallothionein - A Review

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ABSTRACT

Stem Cells are capable of self renewal and can differentiate into other lineages upon induction with specific growth factors and proteins. Mesenchymal stem cells obtained from various sources like adipose tissue, umbilical cord, bone marrow, umbilical cord blood have been reported to have stem cell characteristic features laid down by International Society for Cellular Therapy. Research so far deals with the differentiation and self renewal properties of stem cells, which needs to be understood further. Metallothionein, is a metal binding protein isolated from equine renal cortex and shown to be present in nearly all of our cells, with differential expression of its isoforms. Apart from metal binding properties, this protein expression is deregulated in a variety of diseases and other treatment conditions. With respect to cancer biology, this protein expression is studied in almost all cancer types and results indicate pivotal role of this protein in cancer. Certain cancers are associated with up-regulation while some types are associated with down-regulation. The localization of this protein has also reported to change between actively proliferating and normal cells. Given the importance of this protein in cancer biology, the role of this protein in stem cell renewal as well as differentiation is poorly understood. This review discusses about stem cells, - its type and differentiation; metallothionein – expression, function, review about different cancer types, role in angiogenesis and plausible role of this protein in the self renewal and differentiation of stem cells.

Key Words: Stem cells, Metallothionein, Differentiation, Self renewal, Apoptosis

STEM CELLS

In 1960s, mesenchymal stem cells were first identified as plastic adherent, non-haematopoietic stromal cells in bone marrow with osteogenic potential. Initially they were named as colony forming units and later they were renamed as mesenchymal stem cells (MSCs) as they can readily differentiate into adipocytes and osteoblasts (Pittenger et al., Science, 1999) [1]. Subsequently, differentiation to other lineages like ectoderm and endoderm has also been reported. Since then, MSCs have been isolated from many other tissues like adipose tissue, umbilical cord, umbilical cord blood, umbilical cord Wharton's Jelly, synovial membrane and tooth pulp. In general, stem cells are classified based on their source of origin as embryonic stem cells and tissue specific / adult stem cells. Recently, Shinya Yamanaka reported of creating pluripotent stem cells from terminally differentiated fibroblasts [2-12]. Stem cells are also classified based on their differentiation potential - totipotent (early progeny of the zygote up to the eight cell stage of the morula), pluripotent (Inner

cell mass of embryo, epiblast), multi/oligopotent (Fetal tissues, membranes, placenta and adult stem cells), bipotent (lymphoid or myeloid stem cells) or unipotent (Muscle stem cells) [13, 14].

Characteristic features of MSCs

Ideally MSCs are isolated and identified based on their ability to adhere to static surface; preferably plastic coated with elements which support anchoring of cells and this is considered gold standard method still [15]. Another method of identifying and purifying the MSC population is by sorting of cells via Fluorescence activated cell sorting method. This is done by raising monoclonal antibodies against certain cell surface proteins. These surface markers are said to vary in their expression pattern and are donor-, isolation- and passage-dependent [16]. However there is consensus that MSC do not display CD11b, CD31, CD34, CD45, CD117 and HLA-DR. Positive expression of markers identified so far is: CD13, CD29, CD44, CD73, CD90, CD105, CD166,

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STRO-1, and Sca-1 [17, 18, 19]. Several other methods such as membrane filtration, magnetic beads sorting, capillary electrophoresis, and differential centrifugation have also been employed among other emerging technologies to isolate these cells [20, 21].

MSCs also show multi-potential differentiation ability. Research has shown that MSCs have inherent property to differentiate into cells of mesoderm lineage, forming bone (osteocyte), fat (adipocyte) and cartilage (chondrocyte) cells upon induction [22]. These are the minimal criteria to define the isolated populations of cells as mesenchymal stem or stromal cells as described by the International society for Cellular Therapy (ISCT) in 2006 [16].

Self-renewal and differentiation

Self-renewal may be defined as the ability of cells that sustain the capacity of cells to remain in quiescent or undifferentiated state. It can be influenced by presence or absence growth factors such as Leukemia Inhibitory Factor (LIF), Fibroblast Growth Factors (FGFs), Wnt family of proteins, Sox2 and Oct4 among others. Commitment or differentiation of mesenchymal stem cells, inherently towards mesodermal lineage, as well as cells towards ectodermal and endodermal lineage is a tightly and temporally controlled process guided by microenvironment and culture conditions [23]. These can be regulated in order to enhance the differentiation capacity of MSCs by various methods using biological, biochemical and mechanical approaches [24, 25, 26].

Mesodermal differentiation

Differentiation of MSCs to form bone, fat or cartilage is inherent and can readily be observed upon induction by culturing MSCs with transforming growth factors $\beta 1$ and $\beta 2$ (TGF- $\beta 1$ &2), bone morphogenic protein (BMP)2, 4, 6, 12 and 13. To mimic the bone development in vitro; cells are cultured in the presence of β -glycerophosphate and ascorbic acid-2-phosphate, BMPs, Wnts, dexamethasone which would result in increased alkaline phosphatase activity and calcium deposits and are positive for von Kossa staining. Chondrocytes or cells forming cartilage can be obtained in vitro by culturing cells in high seeding density as pellet or micromass form, in the presence of Insulin-Transferrin-Selenium (ITS), linoleic acid, selenious acid, pyruvate, ascorbate 2-phosphate, dexamethasone and transforming growth factor- β III (TGF- β III). At the end of differentiation period, cells tend to accumulate proteoglycans and type II collagen. To obtain fat cells or mature adipocytes, cells are cultured with dexamethasone, insulin, isobutyl methyl xanthine (IBMX), and indomethacin. The resulted cells are able to produce lipid droplets which can be revealed by addition of oil red stain and expression of Adipocyte-specific genes Peroxisome Proliferator Activated Receptor gamma

(PPAR- γ), Adipocyte protein 2 (ap2) and Lipoprotein Lipase (LPL) genes [17, 27, 28].

Ectodermal differentiation

Despite of mesodermal origin, MSCs have the ability to differentiate into non mesodermal lineage such as neurons and glial cells. Neuronal or glial cells can be obtained upon exposure to cocktails of growth factors like Hepatocyte Growth Factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), neurogenin-1, forskolin, cAMP, β -Mercaptoethanol (BME), Nerve Growth Factor (NGF), Insulin, Retinoic Acid, Valproic Acid, BME, hydrocortisone, Glial cell line derived Neurotrophic Growth Factors (GDNF), Brain-Derived Neurotrophic Factors (BDNF), 5-Azacytidine, isobutyl methylxanthine (IBMX), and indomethacin among others either alone or in combination [29]. Cerebrospinal fluid (CSF) has also been implicated in culture condition to induce neuronal phenotype and function [30].

Endodermal differentiation

Likewise, MSCs of various sources have shown to differentiate into endodermal cell types such as hepatocytes, insulin producing beta cells and renal cells. Trans-differentiation into hepatocytes is a two-step process involving differentiation and maturation [31]. In the presence of EGF, bFGF and nicotinamide, MSCs differentiate into hepatocytes and exposure to oncostatin M, dexamethasone and ITS+ (Insulin, Transferrin, Selenium) premix will form mature hepatocytes which can be confirmed by expression of markers like albumin, α -fetoprotein and nuclear factor 4 α (HNF-4 α). Insulin producing β -cells can be successfully obtained by induction with growth factors, i.e. Acitvin A, sodium butyrate, taurine and nicotinamide. However cells obtained other than mesodermal lineage are yet to be translated in clinical practice due to low efficiency of functional capacity [32].

Though the above mentioned reports have been discussed about the isolation, differentiation and clinical usage of stem cells, research in this field needs more input, particularly with respect to differentiation and clinical application. Metallothionein, is a metal binding protein, expressed in most of the cells and tissues, have a major role in cell division, apoptosis and homeostasis of zinc and copper [33]. Numerous reports were about the role of metallothionein in cancer research [34]. Regarding stem cells, role of metallothionein in self renewal and differentiation needs to be identified.

METALLOTHIONEIN

Margoshes and Valley isolated metallothionein (MT) as a metal binding protein from equine renal cortex. Subsequently many types of MT have been discovered [33]. MT is known to present in all eukaryotes and in some prokaryotes, and

possesses a high degree of homology [35]. Characteristic features of this protein include absence of aromatic amino acid and histidine; presence of highly conserved cysteine residues (cys-x-cys, cys-x-y-cys, or cys-x-cys-cys) in the sequence (where x and y are amino-acids other than cysteine), and the presence of high metal content and a low molecular weight 6-7kDa. It has a high affinity for group I b and II b metals and is found to be a major zinc binding intracellular protein. Zinc and cadmium binds with thiolate clusters and form tetrahedral geometry, where as copper forms trigonal geometry. MT protein contains two domains: N-terminal (beta domain) region with 9 cysteine residues, bind 3 divalent or 6 monovalent ions while the C-terminal (alpha domain) region with 11 cysteine residues, bind 4 divalent or 6 monovalent ions. The two domains are connected by a hinge region composed of a conserved lys-lys segment [36-44]. The MT genes encoding at least 11 MT-I genes (MT-1A, 1B, 1E, 1F, 1G, 1H, 1I, 1J, 1K, 1L, 1X); MT2A, MT3 and MT4 are clustered in the q13 regions of human chromosome number 16 [45]. In mice only one copy of each of MT1, 2, 3, 4 are present within a 60kb region on chromosome 8 [46]. MT-I and II are almost expressed in all tissues, whereas MT-III is restricted to central nervous system and male reproductive organs [47]. MT-IV is expressed only in stratified epithelial cells [47].

EXPRESSION OF METALLOTHIONEIN

The expression of metallothionein was found to be at constitutive levels in most cells but vary with cell type. The expression of different isoforms of MT was found to be regulated differently. The *cis* and *trans* acting factors were involved in the regulation of MT. DNA methylation of *cis* acting elements appears to play a role in the regulation and induction of MT genes in different cell types. The level of *trans* acting factor plays a role in the differential regulation and induction of MT genes in response to heavy metals. Low level, basal level as well as induced level of MT mRNA and protein is controlled by the *cis* acting DNA elements that are located upstream in the 5' region of the MT gene. The classical TATA boxes and the initiator regions (InR) form the promoter regions of MT-I and MT-II. They recruit the transcription factor II-D (TFIID) as a part of the pre initiation complex. Apart from the promoter region, they contain a metal responsive element (MRE), which is also essential for all levels of MT induction. But the MREs act in association with a zinc responsive transcription factor, termed as metal transcription factor-1 (MTF-1). Anti-oxidant Responsive Element (ARE) sequence is also present in adjacent to the MREs. Apart from these, a GC box (consensus sequence GGGCGGGG), which respond to Sp1 transcription factor is also located in the promoter region of MT [48].

FUNCTIONS OF METALLOTHIONEIN:

Metallothionein, which is known to be induced by a variety of factors like heavy metals, glucocorticoids, cytokines, UV rays, hypoxic conditions, oxidative stress, cancer and, play different roles like - acting as metallochaperones to transport metal ions; in maintaining the concentrations of intracellular free zinc and copper ions; in acting as anti-oxidant, scavenging the free radicals; in the detoxification of heavy metals; and in the protection against various stress conditions. In short, metallothionein acts as a primary defense mechanism in the cells in order to protect against various harmful conditions [49].

MT and CANCER

Cellular localization of MT have been reported to be cytoplasmic in non-pathological tissues while in actively proliferating cells, MT localization varies with respect to cell cycle such as in nucleus in the S and G2 phase. This indicates that the altered MT level could correspond to abnormal cell division. Role of MT in proliferation, apoptosis, and invasion in cancer biology have been well studied using biopsy samples or cultured cancer cells. These reports indicate that increase in MT gene as well as protein expression, in actively proliferating normal cells, cancer cells of kidney, breast, lung, nasopharynx, salivary gland, ovary, testes, urinary bladder, leukemia and non-Hodgkin's lymphoma. Decreased MT expression has been reported in human hepatic, prostate and thyroid cancers. Apart from this, reports indicate that MT expression can be used as biomarker to identify the tumor stage [34] [Figure 1].

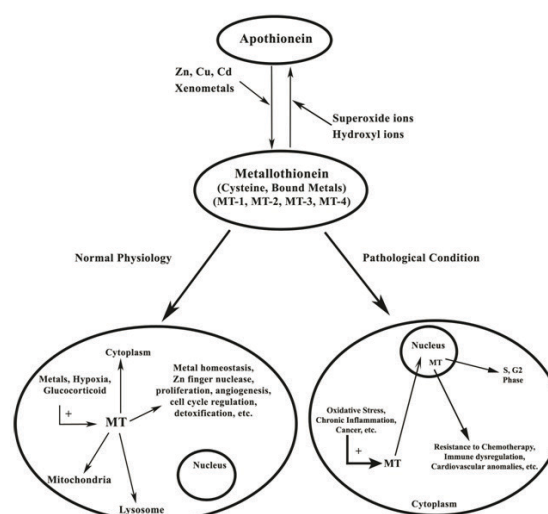


Figure 1: General overview of Metallothioneins (MTs). Thio-nine can exist in two forms intracellularly as Apo-thionein (inactive, unbounded, Cytoplasmic) and Metallothionein (Active, bounded, Cytoplasmic & Nuclear). This transition from Apo- to Metallo- and vice versa is a reversible process. Upon activation, it exerts many physiological functions such as metal homeostasis, gene expression, cell cycle regulation etc.

ENDODERMAL CARCINOMAS AND MT:

Hypermethylation of MT promoter region have been reported in papillary thyroid tissue when compared to normal thyroid tissue, which is associated with down regulation of MT in papillary and follicular thyroid carcinoma. When compared with healthy control tissue, laryngeal tissue biopsies show significant increased expression of MT. Regarding lung cancer, strong MT expression was reported in non-small-cell squamous cell carcinoma and adenocarcinoma while decrease in small-cell lung carcinoma, when compared with healthy control tissue. Reports indicate increased nuclear and cytoplasmic expression of MT in malignant and reactive cells in oral and pharyngeal squamous cell carcinoma. In esophagus carcinoma, MT negative tumors showed decreased lymph node metastases and distant organ metastases when compared with MT positive tumors. Increase in MT expression has been reported in gastric cancers and gastric dysplasia lesions but the levels are unrelated to tumor grade, stage or survival. Nuclei/cytoplasmic expression of MT have been reported in colorectal carcinomas. In hepatic cancer, MT expression is increased in surrounding healthy cells and reactive cells when compared with tumor cells. Regarding pancreatic carcinoma, studies reported that out of 75 patients, 59% showed negative expression and 41% were MT positive. In positive cases, MT expression was localized in cytoplasm and levels were correlated to metastasis and shorter survival. In bladder and ovarian cancer, increased MT staining was associated with tumor stage and grade, reduced survival. There is no association between MT expression and estrogen or progesterone receptor status in ovarian cancer [34] [Figure 2 & 3].

MESODERMAL CARCINOMAS AND MT:

Significant inverse correlation has been reported between MT expression and patient survival in renal cancer. In prostate cancer, increased MT level indicate an improved prognosis. Prostate cancer cells both in in vitro and in vivo show very low expression of MT levels when compared with healthy cells. In contrast, in case of uterine cancer increase in MT expression correlated with higher tumor grade, poor survival and increased tumor cell proliferative capacity [34] [Figure 2 & 3].

ECTODERMAL CARCINOMAS AND MT:

Number of studies reported that MT levels were associated with tumor grade, recurrence rate as well as poor survival in malignant invasive ductal breast carcinomas and MT expression is inversely correlated with estrogen and progesterone receptor status. Regarding skin cancer, mixed results were reported for MT expression. Weinlich et al., (2007) [50] reported that increased MT expression is correlated with poor survival while Suzuki et al., (2003) [51] reported that increased MT expression is protective in initial stages of skin

carcinogenesis. In CNS, increase in MT expression is found to be therapeutically important as it is believed to protect non-malignant astrocytes and neurons from radiation induced apoptosis, thereby increasing the survival [34] [Figure 2 & 3].

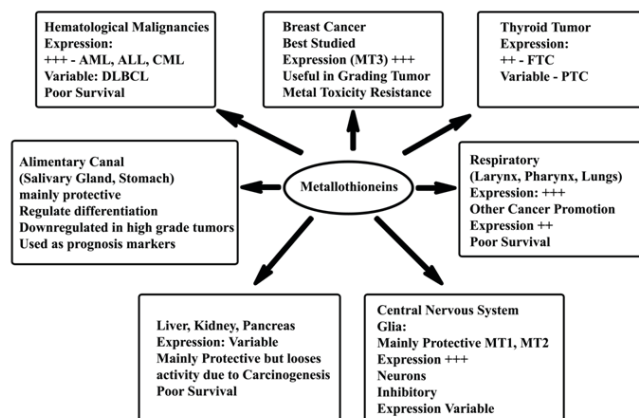


Figure 2: An overview of role of Metallothioneins in various neoplasms and malignancies.

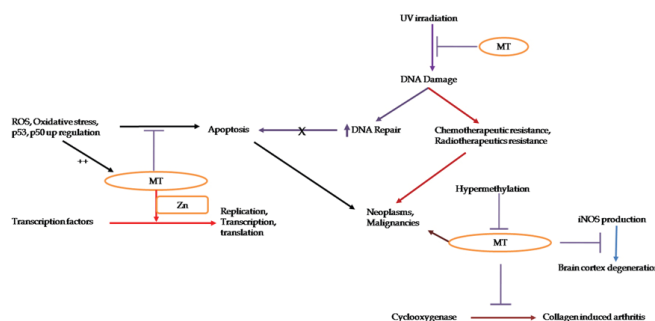


Figure 3: Mechanism of action of metallothioneins (MTs) in physiology and pathology. MTs upon induction by various metals and oxidative stress, translocate from cytoplasm to the nucleus and starts transcription of anti-apoptotic and survival genes by donating the metals, zinc for example, to zinc dependent transcription factors, thus inhibiting apoptosis. However, the same mechanism can lead to abnormal proliferation of cells even in the presence of oxidative stress thereby transforming cells into neoplastic or precancerous cells. MTs also reduce DNA damage caused by UV irradiation and augments DNA repair mechanisms. Conversely, it induces chemo- and radio- therapeutics resistance in the given cell giving them a niche to grow as malignant cell. MTs, on the other hand down regulates/ inhibit iNOS production thus protecting brain cortex degeneration. Also, MTs exert its protective role by inhibiting Cyclooxygenase which causes collagen induced arthritis. Nevertheless, hyper-methylation in the MT genes can diminish its protective activity and thus become a promotor or enhancer of malignant transformation of cells.

REGENERATIVE EFFECTS OF MT:

MT expression during cell cycle varies considerably. In G0 and G1, MT is localized primarily in cytoplasm, while in S and G2 phase, they are seen in nucleus and finally in G2/M, MT expression is cytoplasmic. Apart from this, reports indicate that nuclear expression of MT has a role in cell growth. Augmenting reports indicate that MT can activate transcription factors, metallo-enzymes and cyclin D by which it exerts its function during regeneration and tissue repair. Anti-inflammatory cytokines, growth factors, neutrophils and their receptors like IL-10, TGF- β & its receptor, FGF & its receptor, vascular endothelial growth factor (VEGF), NGF, NT-3-5, brain-derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) were reported to be activated by MT [34].

ANGIOGENESIS AND MT:

Metastasis of cancer requires growth of new blood vessels, supply of oxygen and nutrients to tumor cells. The process of angiogenesis involves degradation of endothelial basement membrane, migration of endothelial cells to perivascular stroma and capillary sprouting. *De novo* synthesis and expression levels of number of growth factors like FGF, TGF- β and VEGF are known to be induced by MT. These factors are known to have a role in angiogenic process. Number of reports indicates relationship between MT and angiogenesis. In MT deficient mice, following the CNS injury, expression of angiogenic factors decreases when compared with that of normal. MT deficiency also inhibits proangiogenic effects of IL-6. *In vivo* expression of MT in endothelial cells at the site of angiogenesis and down regulation of MT in those cells inhibit cell proliferation and migration; *in vitro* network formation as well *in vivo* angiogenesis, have been reported. Also reports indicate that MT down regulation arrest cells in G1 phase. Together these reports indicate that MT has a role in angiogenesis [34].

Stem cells differentiation and Metallothionein:

Dohi et al., (2005) [52] reported that MT-2 expression was increased at both mRNA and protein level during the course of osteoblastic differentiation of MSCs. They further reported that MT-1 & 2 mRNA levels were very high during 48h after addition of dexamethasone and it declined to basal level. The osteoblastic markers alkaline phosphatase (ALP) and osteocalcin mRNA levels increased steadily from day 1 to day 14. Addition of antisense oligonucleotides against MT – 1 & 2 mRNA during the first two days of differentiation in the presence of dexamethasone, decreased the ALP and osteocalcin level after day 14. They reported that early expression of MT mRNA and protein has a role towards osteoblastic differentiation of MSCs. MT as a zinc storage protein inside the cell, might play a role in differentiation process, by controlling the availability of zinc inside the cell.

MT may also directly involve in controlling the differentiation process by interacting with other transcription factors. In 2011, Lin et al., [53] reported that addition of zinc in the differentiation process of dental pulp stem cells towards odontoblasts, augmented the differentiation process. Though they have not confirmed the report by inhibiting MT expression, this report cannot be ruled out regarding the role of MT in differentiation process.

DISCUSSION

Plausible roles of MT in the self-renewal and differentiation of Stem Cell:

MT, Cell cycle and Apoptosis:

Reports indicate that MT localization varies during cell cycle and with cell stage. MT has been reported to be present in cytoplasm in non pathological tissues, whereas in actively proliferating tissues, MT localizes in nucleus. In S and G2 phase, nuclear localization of MT has been reported [34]. Stem cells in their self renewal state can continuously divide. In such case, it will be note worthy to study about the expression, localization as well as role of MT in stem cells. MT has been reported to have role in apoptosis. Particularly when cardiomyocytes from MT transgenic mice are exposed to doxorubicin, MT suppresses apoptosis via inhibition of cytochrome c release from mitochondria and caspase – 3 [54]. Reports also indicate that zinc directly regulates caspase -3 activities [55]. Tumor suppressor gene p53 needs zinc in order to maintain the active structure. Replacement of zinc with cadmium, disrupts its function. Physical interactions between MT and p53 have been reported [56, 57]. Another mitogenic transcription factor NF- κ B was also reported to be deregulated in cancer cells. Reports indicate that MT can interact with p50 subunit of RelA/NF- κ B. This interaction stabilizes the DNA binding activity of NF- κ B which in turn activates expression of several mitogenic genes [58]. Other reports indicate that MT can physically interact with the protein PKC μ , which has dual role in prostate cancer depending upon androgen status. PKC μ expression is repressed in androgen-independent prostate cancer, whereas it is enhanced in androgen dependent prostate cancer. MT is said to directly interact with lysine residue (612) at ATP binding site of PKC μ , which is responsible for its enzymatic activity. This indicates that interaction of MT with PKC μ can decrease its enzymatic activity. This may be a reason for decreased activity of PKC μ in androgen-dependent prostate cancer. However it is note worthy to find out the interaction between MT and androgen [59]. The above reports indicate that MT might have a role in stem cell renewal as well as differentiation. This area of research needs more attention to find out the role of MT in regulating differential genes involved in self renewal as well as differentiation [Figure 4].

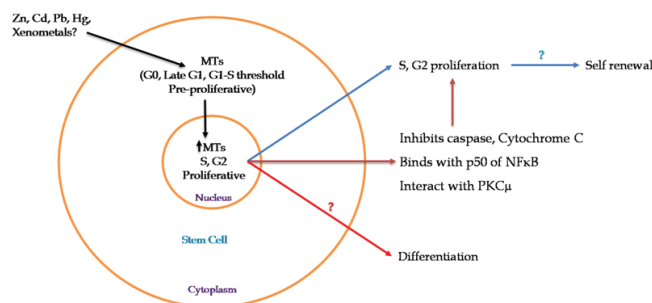


Figure 4: Do metallothioneins have any putative role in stem cell biology? A plausible area of research in the field of regenerative medicine.

CONCLUSION

In a multicellular organism like humans, daily wear and tear process is a common phenomenon. To maintain the cell homeostasis, every organ harbors a small quiescent population of cells termed as stem cells which serve as a reservoir of organ specific progenitor cells. These cells have the ability to both self-renew and give rise to differentiated cells and gain specialized function. Stem cells are reported to be present in various organs and tissues. Stem cell isolation, culture, differentiation and clinical application have been reported by various laboratories. Stem cells have the ability to self renew and differentiate. Research needs more input in understanding the balance between self renewal and differentiation. Though various growth factors and chemicals have been used for maintaining the above mentioned state, role of metallothionein has not been reported in detail. Metallothionein is a metal binding protein, which plays active role in cell division, cancer and apoptosis. This protein is reported to be activated by various agents like metals, growth factors, x-rays, etc. Most of the growth factors and chemicals used in differentiation of stem cells have the ability to activate metallothionein expression. Proteins like p53, NF- κ B and PKC have been reported to interact with metallothionein. These proteins have role in cell cycle and apoptosis. Given the interaction of metallothionein and these proteins, role of metallothionein in stem cell renewal and differentiation cannot be ruled out. This area of research would be helpful in understanding the self renewal and differentiation. By altering the expression of metallothionein, either self renewal or differentiation can be improved, which would yield better population of cells for clinical translation.

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Compliance with Ethical Standards

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