Title Isolation and Culturing of Zebrafish Pluripotent Cells

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ISOLATION AND CULTURING OF ZEBRAFISH PLURIPOTENT CELLS

by

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Masters by Research at the University of Bedfordshire.

It has not been submitted before for any degree or examination in any other University.

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ISOLATION AND CULTURING OF ZEBRAFISH (*Danio rerio*) PLURIPOTENT CELLS

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ABSTRACT

Zebrafish (*Danio rerio*) is an important model organism for the studies of vertebrate development and gene expression in the field of molecular biology and biomedicine. Its embryonic stem cells provide a unique tool linking *in vitro* and *in vivo* genetic manipulations of animal genomes.

The aim of the project was to determine the most suitable embryo stage for the isolation and culturing of pluripotent cells of zebrafish embryos. Studies were carried out to investigate the expression of two pluripotency markers i.e. Oct4 and Sall4 at certain embryonic stages employing Immunohistochemistry. The protein expression studies indicated maximum expression of Oct4 and Sall4 at high stage. Primary cultures were initiated from zebrafish high stage embryos in basal nutrient medium; supplemented with insulin, selenite, epidermal growth factor and Foetal Bovine serum. Experiments were conducted for the determination of optimum concentrations of FBS. The growing cultures identified the signs of differentiation i.e. melanocytes and neurite formation. Basic fibroblast growth factor (bFGF) was found to inhibit the differentiation in cultures. The developed pluripotency markers were tested on cultured embryonic cells. Results indicated that these markers work well for the identification of phenotype of embryonic cell colonies in zebrafish.

The establishment of pluripotent cell line will enable knock out and knock in technologies to be used in this species and have important applications in functional genomics research.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APC	Adenomatous polyposis coli gene
BAD	BCL2- antagonist of death
bFGF	Basic Fibroblast growth factor
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
DAPI	4', 6-diamidino-2-phenylindole
dd H_2O	Double distilled water
EC	Embryonic carcinoma
EGF	Epidermal growth factor
ERK	Extracellular-signal-regulated kinase
ESC	Embryonic stem cells
EVL	Enveloping layer
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FOXD3	Forkhead box D3
GDFs	Growth and differentiation factors
ICC	Immunocytochemistry
ICM	Inner cell mass
IgG	Immunoglobulin G
IHC	Immunohistochemistry
JAKs	Janus-associated tyrosine kinases
LDF	Leibowitz's L-15, Dulbecco's modified eagle's medium and Ham's F12
LIF	Leukaemia inhibitory factor
MEF	Mouse embryonic fibroblasts
Oct4	Octamer-binding transcription factor 4
PBS	Phosphate buffer saline
PI3K	Phosphatidylinositol 3-kinase
RA	Retinoic acid
Sall4	Sal-like protein 4
SMAD	Mothers against decapentaplegic
Sox2	Sry-related HMG box
STAT	Signal transducers and activator of transcription
TGF	Transforming growth factor
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
Upp	Uridine phosphorylase
UTF1	Uundifferentiated embryonic cell transcription factor 1
YSL	Yolk syncytial layer

1 INTRODUCTION

Zebrafish is an important model organism for the studies of vertebrate development and gene function due to its many favourable characteristics. However, one disadvantage of this model system is the absence of embryonic stem cell lines for the efficient production of gene knock outs (Robles et al, 2011). There is not much reported work on the pluripotency markers of zebrafish. However, Robles et al (2011) reported the expression of some candidate pluripotency genes of mouse and human in zebrafish blastomeres at oblong stage and somatic cells. Employing real time PCR the peak expression of Pou, Klf, Sall4 and Hsp60 was demonstrated at oblong stage when compared to adult somatic cells. Similarly relative gene expression results revealed that there is up regulation of pou5 during embryonic development with a down regulation after oblong stage. There have been no studies reported so far on the protein expression of pluripotency markers at different embryonic developmental stages. Robles et al (2011) found the Oct4 protein expression at oblong stage embryos employing immunocytochemistry but their transient ES cell cultures were negative for Oct4 protein which is contradictory to the role of Oct4. Two pluripotency markers Oct4 and Sall4 were selected to analyse their protein expression at different blastula stages to identify the most suitable stage for the isolation of pluripotent cells. The most commonly used zebrafish embryo stage to derive embryonic stem cell cultures is oblong stage (Ma et al, 2001; Fan et al, 2004). The embryonic blastomeres from one cell stage to mid blastula stage of zebrafish embryos are considered to be pluripotent or not yet committed to express a particular fate (Kimmel and Warga, 1986; Ho, 1992; Ho and Kimmel, 1993; Strehlow and Gilbert, 1993). However the established cell lines are blastula derived cell lines rather than embryonic stem cell lines (Xing et al, 2008). A more detailed knowledge of pluripotency specific markers is required for the establishment of bona fide ES cells. Here, studies were carried out on the expression of human and

mouse pluripotency markers Sall4 and Oct4 in zebrafish embryo stages using differentiated cells and embryonic cell cultures.

1.1 EMBRYONIC STEM CELLS

Stem cells are undifferentiated cells that have the ability to continuously divide and differentiate into diverse specialized cells such as blood cells and muscle cells. There are two basic types of stem cells adult stem cells and embryonic stem cells. Adult stem cells are the undifferentiated cells present throughout the animal body and play an important role in replenishing dying cells and regeneration of damaged tissues. Embryonic stem cells (ES cells) are pluripotent embryonic cells derived from the inner cell mass of the blastocyst (an early stage embryo). Under appropriate conditions, ES cells can differentiate into all cell types including germ cells. Therefore, ES cells provide a unique tool linking *in vitro* and *in vivo* genetic manipulations of animal genomes (Hong et al, 2003). Embryonic stem cells are able to give rise after differentiation to derivatives of three germinal layers (ectoderm, endoderm, and mesoderm) and to functional gametes. This property of cells is referred to as pluripotency. Moreover, since ES cells have unrestricted developmental capacity, they represent a promising source for cell transplantation therapies to treat various human diseases (Okita and Yamanaka, 2006).

Mouse embryonic stem cells were successfully derived in early 1980 (Martin, 1981; Evans and Kaufman, 1981). This led to the development of the gene targeting technology used to generate knockout mice, a technique that has quickly become a standard approach for investigating and modelling gene function. Ever since the production of first mouse ES cell line in 1981, various attempts have been made towards the establishment of ES cells in different vertebrate organisms. In 1996 successful ES cell production was reported in medaka (Hong et al, 1996) and subsequently first successful human embryonic stem cell line production was reported in 1998 (Thomson et al, 1998).

The essential features of ESCs are derivation from pre-implantation embryos, pluripotency, capability for prolonged proliferation and self-renewal, a normal euploid karyotype and the expression of distinctive markers (Allegrucci and

Young, 2007). To date, ESCs has been derived from mouse, rat non-human primate and human embryos. ESCs obtained from different species shares many characteristics but there are also some differences.

Although there is an increasing trend towards derivation of ES cells and putative ES like cells (Yi et al, 2010; Hong et al, 2010) and short term ES cell cultures competency of germ line transmission in zebrafish (Ma et al, 2001) have been reported in some species, so far stable ES cell lines production has been limited to few organisms (Wang et al, 2011). One of the main hurdles is the lack of the knowledge of suitable pluripotency markers in these species.

1.1.1 MECHANISM OF PLURIPOTENCY

The mechanism of pluripotency is regulated by a set of key transcription factors and multiple signalling cascades. These regulatory networks play role in maintenance of pluripotent and undifferentiated form of ES cells. However a slight modification in these signals promotes differentiation (Yamanaka et al, 2008). There are numerous signalling pathways (Fig 1.1) that play an important role in maintaining pluripotency during embryogenesis. For instance, the Wnt signalling pathway has important functions in embryogenesis and cancer. TGF-B signalling is essential to maintain ES cell pluripotency, and PTEN signalling plays important roles in the maintenance of hematopoietic stem cell self renewal (Yang et al, 2008).

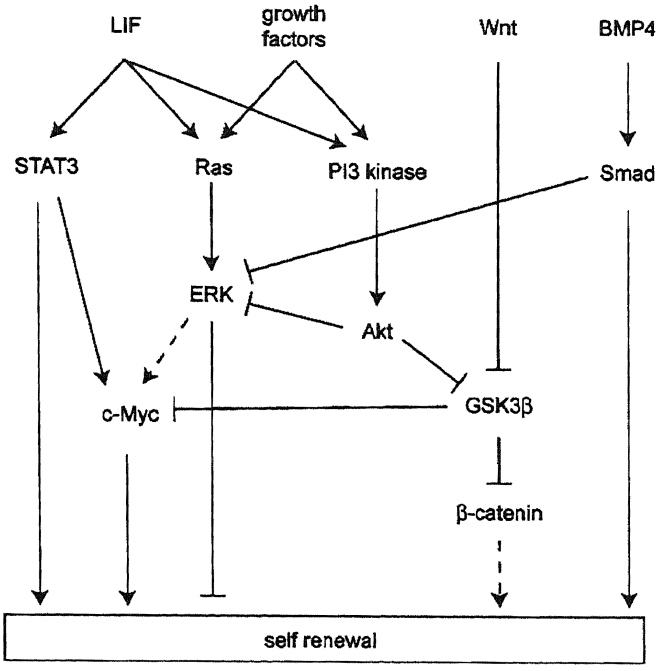


Fig 1.1: Interaction of different intracellular signalling pathways in mouse ESCs. Expression of C-Myc through STAT 3 signal results in the expression c-Myc target genes involved in self-renewal. GSK3β inhibits c-Myc AND β-catenin expression through phophorylation and proteasome-mediated degradation. The suppression of ERK pathway by BMP/SMAD signalling and PI3 kinase pathways favours undifferentiated phenotype of ESCs. Wnt signalling plays role in self-renewal through GSK3 inhibition and resultantly accumulation of β-catenin and c-Myc (Okita and Yamanka, 2006).

1.1.1.1 LIF/STAT3 signalling

Mouse embryonic stem cell can be maintained in a self-renewing state for an indefinite period in the presence of leukaemia inhibitory factor (LIF). The dimerization of cytokine receptors LIF-R and gp 130 results in the initiation of LIF signalling in murine ES cells. The downstream signal is transmitted through gp130. A number of signalling pathways such as STAT3, phosphatidylinositol 3-kinase (PI3K) and Ras/Erk pathways are present downstream of gp130. In STAT (signal transducers and activator of transcription) 3 pathway, non-receptor tyrosine kinases of Janus-associated tyrosine kinases (Jaks) family are activated which subsequently phosphorylate LIF-R and gp 130 on tyrosine residues, which in turn results in the phosphorylation of STAT3. Phosphorylated STAT3

dimerizes and translocates to nucleus and target gene activation. In PI3K pathway, the phosphatidylinositol-3, 4-bisphosphate (PU-3, 4-P2) and phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) are generated through the phosphorylation of phophoinositides on 3-OH position of inositol ring by PI3K. These messengers than activate serine/threonine protein kinase Akt, which phosphorylates its target molecules, glycogen synthase kinase (GSK)-3 and pro apoptotic BCL2-antagonist of death (BAD) protein. In the Ras/Erk pathway, the activation of the Raf/ MEK/ Erk kinase cascade by Ras results in the phosphorylation of Erk target molecules, like Elk-1, pro apoptotic protein capase-9, and p90 ribosomal S6 protein kinase (RSK). Both LIF/STAT 3 and PI3K pathway activation and suppression of Ras/Erk signalling stimulates self- renewal in embryonic stem cells.

1.1.1.2 Wnt signalling

Wnt signalling and Wnt proteins are thought to play numerous roles during development and have been associated with role in stem cell maintenance. It is believed that Wnt proteins transmit their signals to β-catenin through binding to cell surface receptors of Frizzled and LRP families. After the signal transmission, β-catenins forms a complex with TCF in nucleus and activate transcription of Wnt target genes. Wnt signals control the transcription of number of ligands and receptors which express during tissue growth to maintain a balance between proliferation and differentiation. Sato et al (2004) showed that the activation of wnt pathway by 6-bromoindirubin-3′-oxime (BIO), an inhibitor of Glycogen synthase kinase-3 (GSK-3), and its function is to stimulate the proteasome degradation of cytoplasmic β-catenin, helps in the maintenance of both mouse and human embryonic stem cells. There was a normal expression of pluripotent state specific transcription factors Oct-3/4, Rex-1 and Nanog. For the stem cell maintenance it is necessary that the level of several transcription factors including the aforementioned be maintained. Pereira et al (2006) showed TCF3, a Wnt controlled transcription factor, plays role in controlling the expression of Nanog gene in ESCs. Furthermore the activation of β -catenin by inhibiting the expression of adenomatous polyposis coli gene (APC) resulted in the non-differentiative potential of mouse embryonic stem cell (Kielman et al, 2002).

1.1.1.3 The TGF β signalling pathway

The TGF β superfamily of ligands consists of approximately 40 proteins. These proteins transduce signals mainly through two branches. One is SMAD1/5/8 which plays role in signal transduction of BMPs (Bone morphogenetic proteins) and GDFs (growth and differentiation factors) ligands. The other SMAD2/3 branch involves signal transduction of TGF β /Activin/Nodal. Both branches work in association with SMAD 4. The two inhibitory SMADs are: SMAD6 for SMAD1/5 inhibition and SMAD 7 for TGF β signalling. The SMADs after activation by phosphorylation translocate into the nucleus and regulate gene expression in combination with other transcription factors (ShiandJohn, 2003).
James et al (2005) showed the crucial role of TGF signalling in maintenance of human embryonic stem cells. Wnt signalling was found to be involved in SMAD 2/3 activation. Recently a number of studies have shown the prominent role played by TGF signalling in maintenance of hESCs. For example Besser (2004) showed that there were high levels of Nodal, LEFTY1 and LEFTY2 which are controlled by TGF signalling in undifferentiaited hESCs. Similarly role of TGF signalling has been found in the maintenance of hESCs in feeder free conditions (Amit et al, 2004). Furthermore Ying et al (2003) identified that BMP4 is the growth factor which acts in concert with LIF to maintain undifferentiated phenotype of embryonic stem cells in feeder free conditions.

1.1.2 INTRINSIC DETERMINANATS

1.1.2.1 OCT4

Oct4 (Octamer-binding transcription factor 4) also known as oct3 (encoded by pou5f1) is a novel member of POU (Pit-Oct-Unc) family of transcription factors. A distinctive characteristic of POU family is the presence of POU domain. It is comprised of two subdomains one is POU specific (POUs) and the other is POU homeodomain (POU_{HD}), connected through a flexible linker. The binding of POU factors to an octameric consensus sequence motif of an AGTCAAAT results in activation of POU target genes.

Oct3/4 was first identified in murine embryonal carcinoma cells and its expression was found to be strictly limited to pluripotent and totipotent cells (Okamoto et al, 1990; Scholer et al, 1990). The 1.5kb mRNA was found to encode a protein of 377 amino acids and its expression was rapidly down regulated by Retinoic acid (RA) (Okamoto et al, 1990; Scholer et al, 1990). Oct4 was found to be expressed in oocytes, early cleavage stage embryos and the blastocysts of ICM in mammalian embryos (Palmieri et al, 1994) suggesting its vital role in cell pluripotency.

Oct3/4 has been found to play crucial role in mouse embryo development. It was revealed through targeted gene deletion that deficient embryos failed to develop, as the prospective ICM founder cells differentiate into trophectodermal cells (Nichols et al, 1998). A quantitative study carried out by Niwa et al (2000) revealed that a normal expression of Oct4 is necessary for ES cell pluripotency, as an over expression of Oct4 results in the derivation of ES cells to primitive endoderm or mesoderm, while low level of Oct4 triggers dedifferentiation into trophectodermal cells.

1.1.2.1.1 OCT3/4 TARGET GENES

Oct4 as a transcription factor target genes encoding FGF 4 whose expression is restricted to undifferentiated embryonic stem cells and embryonic carcinoma (EC) cell lines in tissue culturing. It is found to be expressed in ICM of blastocysts and later in distinct embryonic tissues (Yuan et al, 1995). Similarly a transcription factor UTF1 (undifferentiated embryonic cell transcription factor 1) expressed in pluripotent embryonic stem cells (Okuda et al, 1998) is also transcriptionally regulated by Oct3/4 (Nishimoto et al, 1999). Likewise an acidic zinc finger protein encoded by Rex-1 (Zfp-42) found abundantly in embryonic stem cells is controlled by oct3/4 activity in Rex-1 promoter (Ben-Shushan et al, 1998) and PDGFαR is also one of the few identified oct3/4 target genes regulated by binding of Oct4 to the promoter of PDGFαR gene (Kraft et al, 1996). Botquin et al (1998) showed by the immunoprecipitation of the first intron of osteopontin (Opn) from cavalenty fixed chromatin of EC sells by Oct3/4 specific antibodies that Opn is

regulated by Oct3/4. Otx-2, Lefty-1, uridine phosphorylase (Upp) and Tera genes expression depend on oct3/4 (Niwa et al, 2000).

Takeda et al (1994) reported the cloning and expression of pou2 in zebrafish. Based on amino acid sequence comparison of POU domains, pou2 was found most closely related to Oct3. In situ hybridization results showed that pou2 transcripts are present from 1 cell stage to gastrula stage. The expression is down-regulated after gastrulation is completed. It was suggested that POU2 acts two times in embryo development. First POU2 acts during early proliferation and morphogenesis of blastomeres the second time it is involved in neuroectoderm development. Burgess et al (2002) suggested that based on chromosomal synteny, phylogenetic sequence comparison and expression and functional data, pou2 is the zebrafish ortholog of mouse Oct3/Oct4 and human POU5F1. Pou2 is closely related to Oct3/Oct4, 79% similarity in POU-specific domain, 74% in POU-homeo domain and 38% in linker region (Takeda et al, 1994). Based on overexpression of truncated t-pou2 by Takeda et al (1994) and morpholino experiments carried out by Burgess et al (2002) it was suggested that pou2 plays its role in maintaining undifferentiated and dividing state of cells and its inhibition resulted in arrest of embryos at gastrula stage. This is very similar to Oct4 role in maintenance of ICM cells in pluripotent state (Scholer et al, 1989; Okazawa et al, 1991; Nichols et al, 1998). In zebrafish embryos the precursor cells of enveloping layer loose pou2 expression during differentiation in early cleavage stages (Hauptmann and Gerster, 1995). Likewise in mouse Oct4 expression is restricted to ICM in developing blastocysts and extra embryonic tissues are deprived of Oct4 (Nichols et al, 1998). Oct4 expression was found in murine neural plate and the injection of murine Oct4 mRNA into spg mutants resulted in the restoration of spg mutant phenotype (Reim and Brand, 2002). However Oct4 alone is not sufficient to maintain pluripotency, as LIF withdrawal resulted mouse ES cell differentiation. This suggests that there must be additional factors involved in pluripotency mechanism.

1.1.2.2 NANOG

Another gene identified to play a crucial role in determining the pluripotent stat of ICM and preventing differentiation of epiblast is Nanog which is a member of Homeodomain proteins (Chambers et al, 2003). Homeodomain proteins are involved in early embryogenesis such as Otx2 and Mixl 1 (Hart et al, 2003). The homeodomain of Nanog shares 50% amino acid similarity with NK2 family of homeopproteins. Nanog is considered to be a unique divergent homeodomain protein. Chambers et al, (2003) and Mitsui et al (2003), through employing different screening strategies, reported the same transcription factor named as Nanog capable of maintaining ES cells pluripotency independent of LIF/STAT signaling. During mouse embryo development Nanog mRNA is first spotted in interior cells of morula than limited to ICM of blastocyst. It is further restricted to epiblast in late blastocyst. After that it is down regulated and detectable amounts are present in germ cells of genital ridges. *In vitro*, Nanog is found to be expressed in all pluripotent ES (murine and human), EG and EC cell lines (Chambers et al, 2003). Nanog expression was found to be down regulated upon differentiation of ESCs. Nanog maintains pluripotency by inhibiting differentiation to extraembryonic endoderm (Mitsui et al, 2003). High levels of Nanog enabled human ESCs to grow in feeder free conditions (Darr et al, 2006) and excluded the dependence of mouse ES cells on extrinsic LIF factor (Mitsui et al, 2003). It suggests that Nanog role is conserved in mouse and humans (Pan and Thomson, 2007).

Suzuki et al (2006) revealed that T (encodes earlier marker of mesoderm differentiation) and STAT3 bind to a site in Nanog 5' promoter region which suggests that Nanog is a downstream effector of a LIF/STAT3 pathway but more investigation is required in understanding the link between Nanog and LIF-STAT3 signaling.

Bone morphogenetic proteins (BMPs) signaling is involved in mesoderm induction during mouse embryonic development and has also been found to play a role in pluripotency maintenance in presence of LIF in ESCs. BMP4 induces Id (inhibitor of differentiation) expression through Smad 1 signaling in ESCs. Suzuki et al (2006) proposed that initially BMPs promote mesoderm differentiation which

results in up regulation of T (Brachyury). Both T and activated STAT3 bind to Nanog promoter and stimulate its high expression. Elevated levels of Nanog than physically interacts with Smad 1 and blocks the mesoderm progression and maintains undifferentiated phenotype of ESCs.

Nanog 5' promoter region contains a composite element, found to be binding site of Oct4 and Sox2. Oct4/Sox2 binding to this element resulted in regulation of Nanog transcription and this element is fully conserved among Eutherian mammals. Rodda et al (2005) demonstrated binding of Oct4/Sox2 binding to Nanog promoter *in vitro* and *in vivo* through electrophoretic mobility shift and Chip assays and is indeed involved in activity of Nanog in pluripotent cells (Kuroda et al, 2005). In situ hybridization showed that Nanog mRNA is detectable in Oct4 deficient embryos (Chambers et al, 2003), suggesting other transcription factors in addition to Oct4 are involved in Nanog regulation. Pan et al, (2006) identified FOXD3 as an activator of Nanog. Oct4-Nanog-FoxD3 was found to form a negative feedback loop for maintenance of stem cells self-renewal and pluripotency (Fig 1.2). Likewise during embryonic development Nanog needs to be down-regulated. During ES cell differentiation the down regulation of Nanog levels was found to be related to high transcriptional activity of p53 (Lin et al, 2004).

Schuff et al (2011) identified Nanog ortholog in zebrafish and this was found essential for zebrafish embryo development as its deficiency led to gastrulation defects and lethality. They found that mouse Nanog overexpression resulted in overcoming of these defects. In the same way zebrafish Nanog played same role as of mouse Nanog of proliferation and pluripotency in mouse embryonic stem cells (Schuff et al, 2011).

1.1.2.3 SOX2

Sox2 belongs to SOXB1 subgroup of sox gene family. Sox gene family encodes transcription factors with one HMG (high mobility group) DNA binding domain. Based on homology within and outside HMG box, Sox1 and Sox3 are members of SOXB1 group. Sox2 RNA was first identified in morula cells, then in ICM and

epiblast, extra embryonic ectoderm and later in germ cells. Sox2 expression is also associated with developing CNS (Avilion et al, 2003). *In vitro* Sox2 in combination with OCT3/4 stimulate oct-sox enhancer activity. Masui et al (2007) showed that Sox2 is optional for this activity and forced expression of Oct4 in Sox2 null mouse ES cells resulted in restoration of pluripotency. This indicates that Sox2 plays its role in pluripotency through stabilizing the levels of Oct4 in ES cells. Fong et al (2008) revealed role of Sox2 in human embryonic stem cell pluripotency and self-renewal. Using RNA interference they demonstrated that knockdown of Sox2 resulted in increase of trophectodermal markers expression (CD9, Cdx2, GCM1, hCG, HLA-G, hPL, CK7, and SSEA1) and decrease in stem cell markers including Oct4 and Nanog. Furthermore down-regulation of Sox2 resulted in decreased levels of Oct4 and Nanog to 14% and 17%. This further confirms that Sox2 plays a critical role in pluripotency network (Boyer et al, 2005; Rodda et al, 2005).

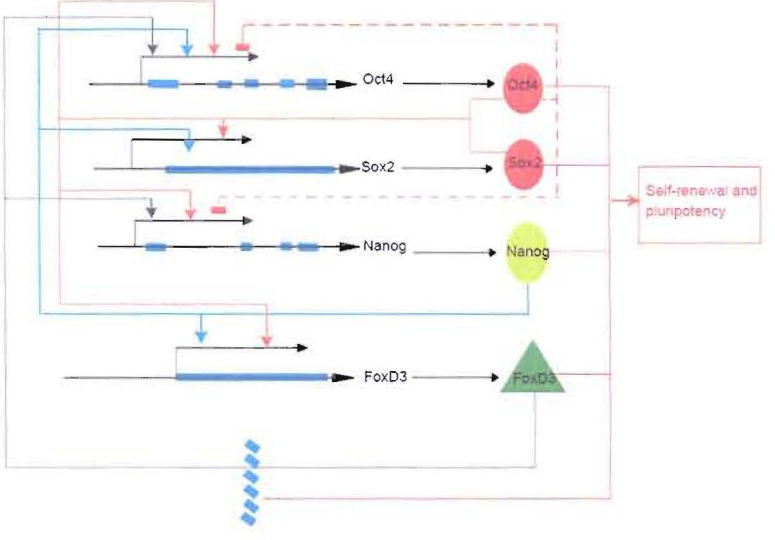


Fig 1.2: Regulatory network of key transcription factors in maintaining ES cell pluripotency and self-renewal. Regulators such as Oct4, Nanog, Sox2 and FoxD3 bind to each other's promoter and support or limit each other's expression, forming an interconnected auto regulatory network to maintain ES cell pluripotency and self-renewal. Arrows connected to factors by solid lines indicate positive regulation of a promoter by the factors. Broken lines linking to Oct4 indicate negative regulation (Pan and Thomson, 2007).

1.1.2.4 SALL4

The SALL gene family consists of four genes Sall1, Sall2, Sall3 and Sall4. It was originally cloned based on a DNA sequence homology to the Drosophilla gene Sall. Sall gene family plays an important role in development. In humans mutation in sall1 results in Townes-Brockes syndrome in patients indicating renal, limb, anal and ear malformations (Kohlase et al, 1998; Powell and Michaelis, 1999). In mice sall 1 null mutant die perinatally because of severe kidney dysgenesis or agenesis (Nishinakamura et al, 2001). No human congenital malformation has been associated with Sall2 so far. It is proposed that Sall3 which maps to chromosome 18q23 is involved in the phenotype of patients with 18q deletion syndrome, which shows the symptoms of developmental delay, hypotonia, growth retardation, mid face hypoplasia, hearing loss and tapered fingers. Sall3 null mice exhibit plate deficiency, abnormalities in cranial nerves, and perinatal lethality. In human Sall4 is found to be mutated in patients with Duane Radial Ray syndrome (Al-Baradie et al, 2002) and a range of overlapping phenotypes includes Holt-Oram, Acro-renal-ocular syndrome, and IVIC syndrome (Kohlhase et al, 2003; Paradisi and Arias, 2007).

In addition to the role in development Sall gene family has found to play an important role in human and murine embryonic stem cells and in early embryonic developments. Sall4 (Sal-like protein 4) has been found to express at 2 cell stage similar to Oct4.

In humans there are two Sall4 isoforms Sall4A and Sall4B. Yang et al (2010) reported that both Sall4 isoforms trigger the Oct4 expression but suppress those of Sall1 and Sall3. In turn Oct4 was found to activate the expression of Sall4, Sall1 and Sall3, which suggests that a positive transcriptional feedback loop exists between Sall gene family members and Oct4. Both Sall4 isoform can inhibit its own promoter in a dose dependent manner and Sall4 activation by Oct4 is determined by Sall4 expression level. Conclusively Sall4 expression is strongly regulated by self-repression and a positive feedback from Oct4.

In murine ES cells, Sall4 has been found to play a fundamental role in maintaining pluripotency and through transcriptional regulation of Oct4 and Nanog governs the decisions involving the fate of ES cells. Sall4/Oct4/Nanog transcriptional core network is crucial for the maintenance of 'stemness' of ES cells and Sall4 has been shown to trigger Oct4 and interact with Nanog (Yang et al, 2010). Fig 1.3 shows the no. of genes co-occupied by these proteins. It shows a total number of 48 genes are co-occupied by Oct4, Sall4 and Nanog. Under appropriate culture conditions reduction in Sall4 levels in mouse ES cells results in re specification of ES cells to trophoblast lineage. Sall4 regulates transcription of pou5f1 which encodes Oct4, by binding to the highly conserved regulatory region of pou5f1 distal enhancer and triggers pou5f1 expression *in vivo* and *in vitro*. Hence it shows that Sall4 by regulating Oct4 expression plays an essential role in maintenance of ES cell pluripotency. It also plays an important role for early embryonic cell fate decisions (Zhang et al, 2006).

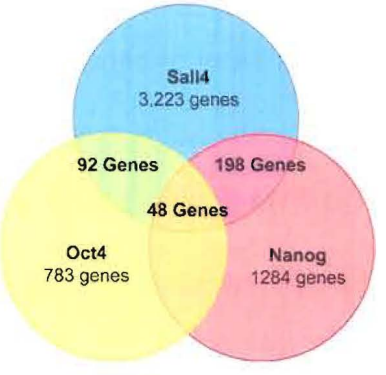


Fig 1.3: Venn diagram showing the overlapping target genes of Sall4, Oct4, and Nanog as determined by ChIP-Chip and ChIP-PET experiments. These complexes may function in the regulation of stem cell pluripotency (Yang et al, 2008).

1.2 APPLICATIONS OF EMBRYONIC STEM CELL CULTURES

The remarkable characteristic of pluripotency of embryonic stem cells has many important applications. The generation of mouse ES cell lines has enabled the investigation of gene function through gene targeting technology (Capecchi, 1989). ES cells can contribute to germ cell lineage after transplantation into host embryos so they can be utilized as vectors for production of transgenic animals.

This is advantageous over conventional transgenesis methods, as the cells can be manipulated *in vitro* and colonies can be selected on the basis of their desirable transgene expression (Collodi, 1996). *In vivo* analysis of roles of individual genes during embryo development and growth was made possible due to mouse knock out production (Nasevicius and Ekker, 2000). The directed dedifferentiation of ESCs to specific cell types such as cardiomycetes or neurons will both serve as a source of drug discovery and transplantation therapies for Parkinson disease, neurodegenerative diseases and myocardial infarction etc (Thomson et al, 1998). The availability of ES cell system will enable manipulation of ESCs to quantify the effect of drugs. They have already been used in studying the role of specific genes, in identification of progenitors involved in hematopoietic, vascular, and neural development (Keller, 2005).

Although the properties of embryonic stem cells make them a promising source in cell replacement therapies there are still a number of obstacles which are keeping them away from their clinical applications e.g. their pluripotency results in the formation of haphazard cells leading to teratomas and teratocarcinoma formation *in vivo* (Wu et al, 2007).

1.3 ZEBRAFISH AS A MODEL ORGANISM

Zebrafish was first used as a model by Dr Georges streisinger, a scientist working in developmental genetics at the University of Oregon, Washington, USA (Westerfield, 1993). Zebrafish, *Danio rerio*, a tropical fresh water fish belong to Cyprynidae family and it is native to Pakistan and India. Among teleosts, the zebrafish is an important experimental animal in developmental biology, physiology, genetics, and biomedical research (Buono and Linser, 1992; Squire et al, 2008). Zebrafish exhibit many features that make them an ideal genetic model organism as they are easy to maintain and manipulate in the laboratory. Zebrafish has a relatively short generation time of 3-4 months and produces a large number of embryos throughout the year making it feasible for large scale genetic screening. The optically transparent embryo develops *ex vivo* making them well-situated for a wide range of experimental manipulations (Gerhard, 2003). As zebrafish is a vertebrate organism, it shares a high genomic, proteomic and

metabolomic homology with other vertebrates and was termed as "canonical vertebrate" for its high similarity with mammalian biology. Studies have been carried out demonstrating that zebrafish genome can be used for the analysis of genes homologs of humans (Rubinstein, 2003). The work carried out by Karlovich on Huntington disease gene homolog in zebrafish (Karlovich et al, 1998), similarly cloning of zebrafish homologs of human enzymes involved in steroidogenesis (Lai et al, 1998) and cloning of zebrafish gene involved in Alzheimer's disease (Leimer et al, 1999), supports the use of zebrafish as model of human diseases studies. The studies on zebrafish heart, vasculature, blood and kidney development were found to be significantly relevant to medicinal studies (Ackerman and Paw, 2003).

1.3.1 NEED FOR ZEBRAFISH EMBRYONIC STEM CELLS

In the last two decades remarkable progress has been achieved with culture of murine and human embryonic stem cell lines. However there is very limited report in the literature on fish stem cell culture (Xing et al, 2008). The production of mouse knock outs using pluripotent ES cell cultures has served as a valuable tool in study of gene function during embryogenesis and growth (Ekker et al, 2001; Weinholds et al, 2002), but cell mediated gene transfer has not been achieved in this specie. The main reason for this is the non-availability of zebrafish ES cell lines that remain germ line competent for multiple passages (Wong et al, 2006).

There is a continuous effort being made to establish zebrafish embryonic stem cell lines and reports have been published on the derivation of pluripotent ES cells, e.g. there are reports demonstrating the germ line competency of zebrafish cell cultures (Fan et al, 2004) and homologous recombination in these cultures (Fan et al, 2006). However, despite these great advances, only zebrafish blastula cell lines have been established (Xing et al, 2008).

1.3.1 DEVELOPMENTAL STAGES OF ZEBRAFISH EMBRYOS

Zebrafish development is show in Fig 1.4. The main key events taking place during development are:

Zygote Period: the newly fertilized egg enters zygote period after first cleavage, which takes place app. 40 minutes after fertilization. During this period, cytoplasm streams toward animal pole to form blastodisc.

Cleavage period: this period lasts for around 2 hours and consists of 2-cell stage to 64-cell stage. During this phase 6 cleavages occur and cells or blastomeres divide synchronously at 15 minute interval. The cytoplasmic divisions are meroblastic that means that blastomeres remain connected through cytoplasmic bridges.

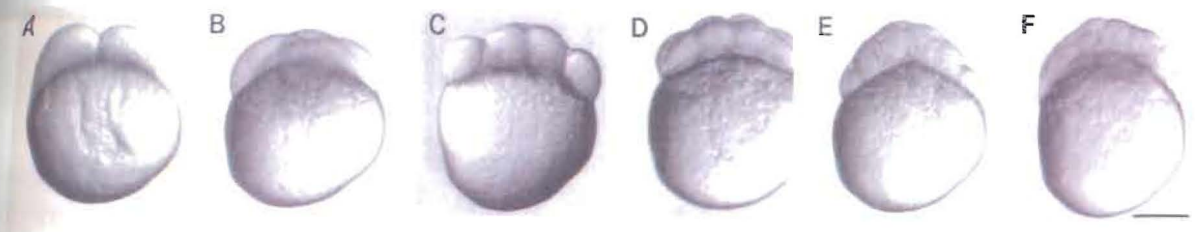
Blastula period: this period covers 128-cell stage to 30% epiboly stage and lasts from 2h 15m – 5h 15m. During this period, blastodisc appears like a ball shape. The main events are; embryo enters (mid blastula transition) MBT at 10^{th} cleavage i.e. is 512-cell stage, the embryo is formed of three layers: the enveloping layer (EVL), deep cells and the yolk syncytial layer (YSL), and epiboly begins, characterized by spreading of YSL and blastodisc over the yolk cells, and reaches 30% of distance between the animal and vegetal poles.

Gastrula period: this period ranges from 5h 20m – 10h and covers 50% epiboly to bud stage. During this phase epiboly continues and cell movements of involution, convergence and extension results in the formation of primary germ layers i.e. epiblast (later gives rise to endoderm) and hypoblast (that will form a mixture of ectoderm and mesoderm) and the embryonic axis.

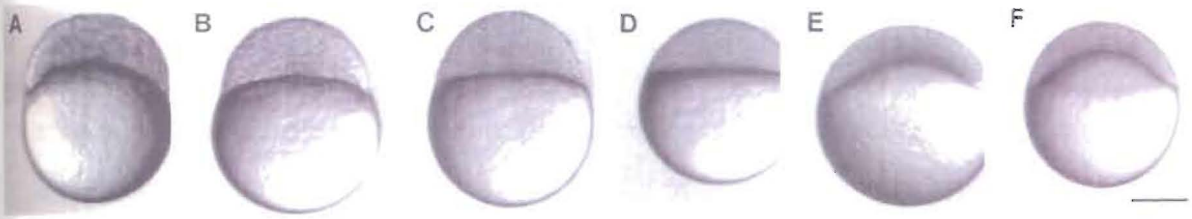
Segmentation period: it lasts for 10h -24 h and covers 3-26 somite stage. Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis and appearance of tail are marked features during this period.

Pharyngula Period: this period covers Prim-6- High pec stage and lasts up to 24h to 48h. The body axis straightens from its early curvature about the yolk sac; circulation, pigmentation and fin development begins.

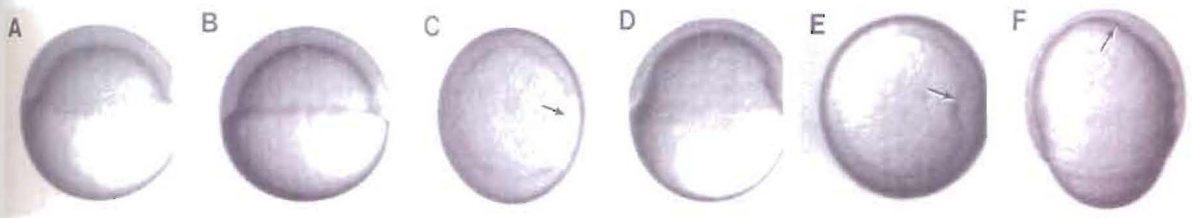
Hatching period: it is the last period and comprises of Long pec- protruding mouth and continues from 48h-72h. This phase is marked by morphogenesis of primary organ systems, cartilage development in head and pectoral fin and hatching occurs asynchronously (Kimmel et al, 1995).



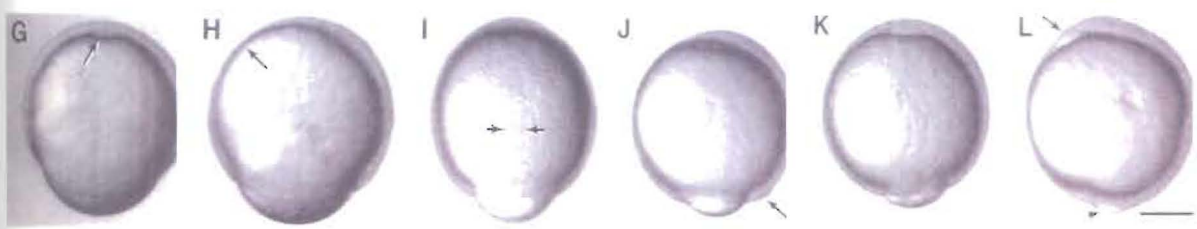
A: 2- cell stage B: 4-cell stage C: 8-cell stage D:16-cell stage E: 32-cell stage F: 64-cellstage



A: 256 cells stage B: High stage C: Oblong stage D: Sphere stage E:Dome stage F: 30% Epiboly



A:50% epiboly B: Germ ring s C: Germ ring (Animal pole view) D: Shield E: Animal pole view F: 70% epiboly



G:70% epiboly stage H: 75% epiboly stage I: 80% epiboly stage J: 90% epiboly stage K: 90% epiboly L: Bud stage

Fig 1.4: Embryonic Development stages of zebrafish (Cleavage, Blastula and Gastrula Period) (Kimmel et al, 1995).

1.4 CELL CULTURING

Cell or tissue culturing, the cultivation and growth of cells *in vitro*, has become an important tool in biomedical sciences. There is an increased use of *in vitro* cell culturing in basic cell and molecular biology research and other disciplines such as pharmacology, toxicology and physiology. There is a need of appropriate culture environment that represents the physiological condition of cells *in vivo*, for successful growth and propagation of cells (Davis, 2002; Masters, 2000). The micro-environment of cells is made up of three components (Fig 1.5); the culture substratum i.e. is the culture dish or specifically coated surfaces for cell attachment, the culture medium consisting of all types of nutrients and cell to cell interaction (Gastraunthaler, 2003).

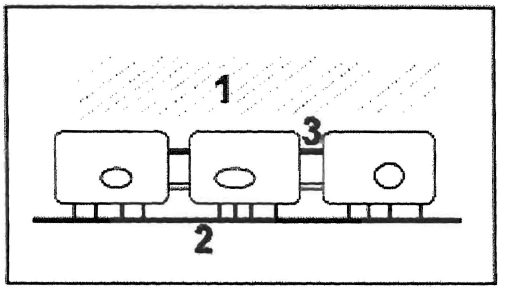


Fig 1.5: Parts of the microenvironment of the cell *in vivo* and *in vitro*. (1) the diffuse environment, *in vitro* provided by culture medium, (2) the contact environment (cell-matrix attachment), (3) the junctional connections between neighbouring cells (cell-cell adhesion) (Gastraunthaler, 2003).

1.4.1 EMBRYONIC STEM CELL CULTURE

Embryonic stem cell cultures are derived from the inner cell mass (ICM) of the early embryo blastocysts. There are two approaches for obtaining ICM derived cells. The first technique involves the transfer of whole preimplantation blastocysts into tissue culture. After hatching, embryos attach to culture dish by trophectoderm and subsequently ICM is exposed after trophectoderm spread over the culture surface. ICM derived cells are allowed to grow for additional few days till they are dissociated and replated (Evans and Kaufman, 1981; Robertson, 1987). The second technique involves the immunosurgical removal of ICM from

blastocyst by selective trophectoderm lysis (Solter and Knowles, 1975; Martin, 1981) or treatment with calcium ionophore A23187 (Surani et al, 1978).

The isolated ICM are then transferred to culture dish containing appropriate medium in presence of feeder layers and allowed to grow for a few days. The cell aggregates are collected before they are getting too large and dissociated in trypsin/EDTA and transferred onto a new plate with feeder layer. After 2 days in culture colonies are apparent. Pluripotent cell colonies possess homogenous appearance while differentiating colonies exhibit fibroblastic, epithelial or trophoblast -like morphology. After approximately 7 days colonies exhibiting stem cell morphology are collected, dissociated and transferred to fresh feeder layers. The cells are grown to confluence (Collodi, 1996).

These are the methods described for derivations of murine embryonic stem cells and with minor modification are used for culturing of stem cells of other species.

1.4.2 FOETAL BOVINE SERUM

Foetal bovine serum (FBS) is derived from bovine foetuses through cardiac puncture from pregnant cows during slaughter. It is widely used in cell culture due to its high content of growth stimulatory factors and low contents of growth inhibitory factors.

The rich content of growth factors and low levels of gamma-globulin as compared to other animal sera (Gastraunthaler, 2003) has made FBS the standard medium supplement. There are certain advantages in using FBS in culture medium; FBS is a combination of growth factors required for cell growth and maintenance, it is also considered as universal growth supplement for a variety of both human and animal cells.

However there are a number of disadvantages associated with its use in cell culture. FBS is chemically undefined (Bjare, 1992) and can be a potential source of contaminants such as microbial. Also there is a batch to batch variability due to variations in the serum compositions (Price and Gregory, 1982).

1.4.3 GROWTH FACTORS

A diverse group of polypeptides that modify cell proliferation are growth factors. They differ somewhat from classic hormones as neither their synthesis site nor their action site is restricted to defined tissues. Many growth hormones work in paracrine fashion and in certain instance they can be autocrine.

1.4.3.1 EPIDERMAL GROWTH FACTOR

There is a complex network of growth factors and receptors that cooperate to modulate the growth of cells. Epidermal growth factor is a part of this complex network. After the release of EGF from cells, it can be either picked up by the same cell or by the neighbouring cell resulting in stimulation of their ability to divide and grow. There are receptors present on the surface of the cell which bind to EGF and transmit the signal inside (Fig 1.6) (Goodsell, 2003).

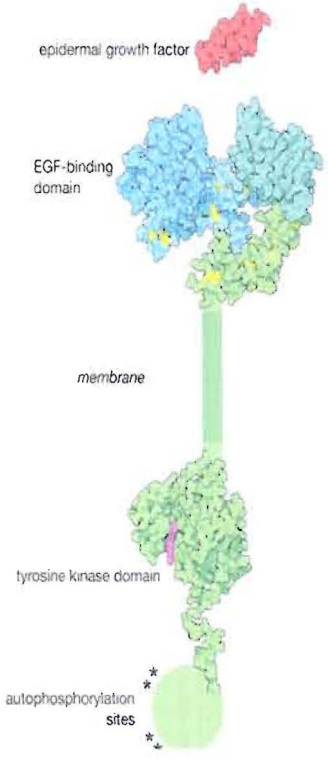


Fig 1.6: In an unbound state, EGF receptor folds into an inactive form that cannot dimerize. Yellow color indicates cysteine amino acids in EGF binding domain. EGF binds in the groove at the top. The purple color in tyrosin kinase domain indicates drug (Goodsell, 2003).

After binding with EGF, receptor is activated by forming a dimer with other receptors. Four related receptors have been discovered: the EGF receptor and three variants. These receptors may dimerize with themselves or may form heterodimers with other types. A varied no of set of growth factors interacts with these receptors. These include for example EGF, transforming growth factor-alpha (Shawver et al, 2002). The receptor is made up of a single chain with many fuctional parts. It is located in the cell membrane with one portion facing out to receive the signal and the other facing in to transmit the message to cell machinery (Fig 1.7). The outer portion of the receptor form the EGF binding domain, comprised of four articulated parts: two globular parts that holds the EGF and two rigid rod shaped linkers. After binding of EGF to this multi-part domain, it changes its shape relaesaing one of the long cysteine-rich sections, allowing receptor to dimerize with others (Goodsell, 2003).

The inner portion of the receptor is tyrosine kinase enzyme. The kinases add phosphate groups to tyrosine residues on the neighboring chain. The signaling proteins present inside the cell than bind to these phosphorylated tyrosinases, inducing the signaling cascade that ultimately initiates DNA synthesis and cellular growth (Schlessinger, 2000).

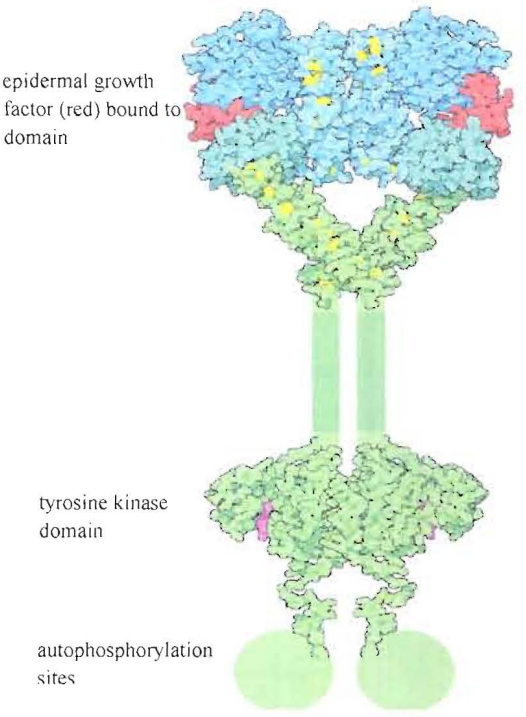


Fig 1.7: EGF binding to its receptor followed by change in recceptor shape resulting in receptor dimerization. This brings the kinase domains closer allowing addition of phosphate groups to each other, resulting in activation of signalling process (Goodsell, 2003).

1.4.3.2 FIBROBLAST GROWTH FACTOR

Originally FGF was identified as an activity that stimulates the growth of 3T3 cells in extracts of pituitary and brain. The activity was found to be due to two proteins (Armelin, 1973; Gospodarowicz, 1974). One of them was aFGF that had an acidic pI and eluted from heparin sepharose with 1MNaCl (Maciag et al, 1984, Thomas et al, 1984), the second was bFGF with a basic pI and eluted from heparin sepharose with 1.5M NaCl. bFGF shares 55% sequence homology to aFGF (Esch et al, 1985).

Fibroblast growth factors comprise a large family of polypeptide growth factors consisting of 23 members who are structurally related and transduce signal through receptors tyrosine kinases. During embryonic development FGFs play crucial role in invertebrates and vertebrates (Bo¨ttcher and Niehrs, 2005). *In vitro* FGFs act as mitogen for mesoderm and neuroectoderm- derived cells and *in vivo*

found to play role in angiogenesis (Montesano et al, 1986). Bouche et al, (1987) showed that during the transition of cells from G_0 to G_1 phase bFGF penetrates the cell and start localizing in nucleolus and complemented by pre-rRNA synthesis. It is one of the structured events taking place in the cells entering from dormant to growing stage (Pardee et al, 1985) and supports the role of bFGF in cell proliferation.

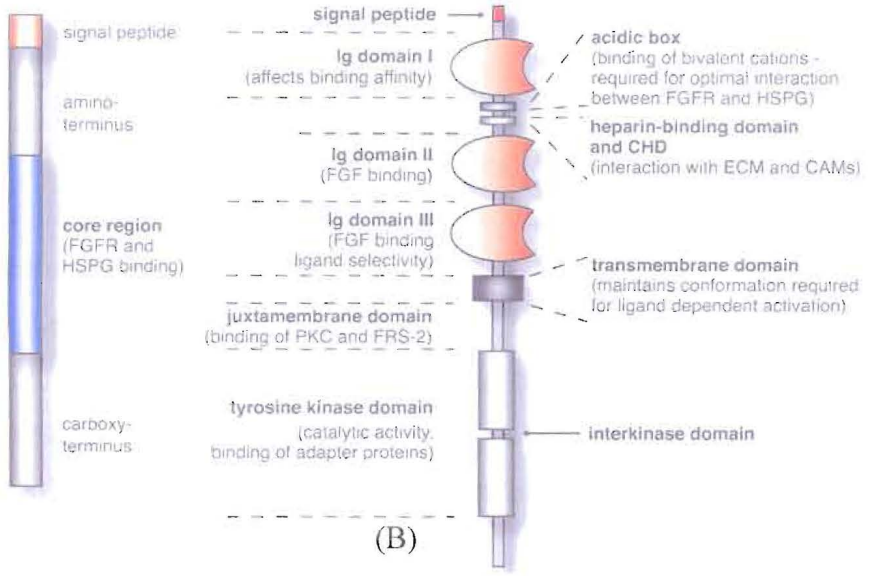


Fig 1.8: Domain structure of FGF and FGFR proteins. (A) Structure of a generic FGF protein consisting of a signal sequence, and the conserved core region consisting of receptor- and HSPG-binding site. (B) The main structural features of FGFRs including Ig domains, acidic box, heparin-binding domain, CAM-homology domain (CHD), transmembrane domain, and a split tyrosine kinase domain are illustrated with respective functions. CAM, Cell adhesion molecule; ECM, extracellular matrix; PKC, protein kinase C (Bottcher and Niehrs, 2005).

FGFs transduce signals through binding and activation of FGFRs. The receptor is composed of single transmembrane protein with extracellular ligand- binding region for FGF and intracellular domain harbouring tyrosine kinase activity. The receptor dimerization due to binding of FGF activates tyrosine kinase and resultantly auto phosphorylation of the intracellular domain.

Xu et al (2005) confirmed that bFGF in combination of other growth factors supports the growth of ESCs in feeder free conditions. Furthermore it was found that BMP antagonist Noggin in concert with bFGF plays role in maintaining undifferentiated phenotype of hESCs in absence of (MEF) mouse embryonic fibroblasts (Wang et al, 2005; Xu et al, 2005). *In vitro*, bFGF reduced the expression of neural-specific markers in zebrafish embryonic cells possibly indicating that it suppresses early neural cell development (Singh et al, 2001).

1.5 IMMUNOCYTOCHEMISTRY

Coons et al in 1941 reported the use of an immunoflourescense technique for the detection of cellular antigens in tissue sections; this was the beginning of immunohistochemistry (IHC). Basically IHC technique encompasses three scientific fields: immunology, histology and chemistry. The process involves the detection of Antigents (Ag) within tissue sections by means of specific antibodies (Abs). The antigen-antibody (Ag-Ab) binding is evident by a colour histochemical reaction visible by light microscopy or fluorochromes with ultraviolet light.

Earlier attempts included the use of specific antibodies labelled with fluorophore itself and applied to the biological samples for the identification of antigenic sites known as direct method. Later more sensitive and versatile indirect method was introduced which employed a secondary antibody tagged with a fluorophore (FITC, TRITC and rhodamine) or enzyme to detect the specific antibody attached to the antigen of interest.

Immunofluorescence in combination with confocal microscopy is a powerful technique for studying of location of proteins and dynamic processes. It is widely employed in cytology and pharmacology fields in disease diagnosis and drug development (Ramos-Vara, 2005). It has become a vital tool in research and diagnostic laboratories.

1.6 AIMS OF STUDY

The objectives of this study were:

1. Development of suitable pluripotency markers in zebrafish, by analysing the expression of different pluripotency genes employing immunocytochemistry.
2. Identification of most suitable stage for isolation of pluripotent cell of zebrafish embryo.
3. *In vitro* culturing of zebrafish pluripotent cells in a simplified medium.
4. Application of pluripotency markers in cultured zebrafish embryonic cells.

2 METHODS ANDMATERIALS

2.1 GENERAL METHODS

All experiments were carried out in the laboratories at IBEST institute, University of Bedfordshire. There is not much reported work on behaviour of pluripotency markers in Zebrafish embryonic cultures (Robles et al, 2011). This can be one of the hurdles towards the successful establishment of Zebrafish ES cell lines. So, studies were carried out on the development of pluripotency markers in zebrafish species. Experiments were conducted for the analysis of protein expression of candidate pluripotency markers i.e. Oct4 and Sall4 of mouse and human at different Zebrafish embryo stages. These studies led to the identification of suitable embryonic stage for derivation of ESCs. Further, the application of these pluripotency markers was analyzed on cultured embryonic cells.

2.1.1 ZEBRAFISH MAINTENANCE

Adult Zebrafish (Fig 2.1) was provided to IBEST by Aquascope, LTD (Birmingham, UK). They were maintained in 30 litres tank (28x 28x58 cm) at 28°C, with tap water which was aged 12- hrs before use. There are temperature control and filtration systems in each tank. Males and females were kept together with a ratio of 1:2 (25 to 30 fish in each tank). The fish tanks were cleaned weekly with and over 80% of the water changed weekly with complete renewal every 4 weeks. Automatic light cycle control was set for light/dark = 12/12h.

Fig: 2.1: Adult males and females Zebrafish. Picture adapted from (http://www.cosmosmagazine.com).

2.1.2 FEEDING

Adult fish were fed three times a day with Tetra Min flakes fish food produces by Tetra, Germany. To enhance their breeding process the fish were also fed once a day with live adult brine shrimps. Excess of the food in the water was removed. A holiday tablet fish food was put inside the tank at weekends and holiday periods.

2.1.3 BREEDING AND EMBRYO COLLECTION

When female fish reach three months of age and the average length and weight of 24.9mm and 1100mg respectively, they are ready to start spawning. Each female fish spawns between 200 and 600 eggs per event. Zebrafish breed daily shortly after dawn, or after light is turned on in captivity. Embryos were collected from a glass tray covered by a plastic net. Embryos were then kept in beakers at 28 °C water tank until they reach the desired stage.

2.2 DECHORIONATION AND DEYOLKING OF ZEBRAFISH EMBRYOS AND ISOLATION OF BLASTOMERES

The experiments for cell culturing were carried out using high stage embryos (3.3hpf) as previous studies have demonstrated that blastula derived cell lines were derived from oblong stage embryos (Xing et al, 2008).

Once the embryos reached the appropriate stage for experiments (Fig 2.2) (high stage) for cell culture, and (128-cell stage, high stage, oblong stage, dome stage and 50% epiboly) for pluripotency markers, dead and unfertilized embryos were removed. Viable embryos were placed in a 6 well plate and tap water was replaced with embryo medium. Embryos were rinsed several times with water and then rinsed with LDF medium (50% Leibowitz's L-15, 35% Dulbecco's Modified Eagle's Medium, and 15% Ham's F12) three times. After LDF removal embryos were washed with bleach solution (0.5% in ddH_2O) for no more than 2 minutes. Embryos were then immediately rinsed with LDF medium. Bleach treatment was repeated twice and rinsed two additional times with LDF medium. Embryos were dechorionated with incubation in 0.5mg/ml pronase solution until the chorions start breaking apart. Chorions were also removed mechanically with sharp forceps and needles. Dechorionated embryos were placed in embryo medium. Embryos were deyoked with needles and yolk gets dissolved in embryo medium. Separated blastomeres were than washed in LDF medium. Blastomeres were then transferred to 1.5ml eppendorf tube. Blastomeres were dissociated with gentle pipetting. Cells were collected by centrifugation at 1500g for 5minutes. Number of embryos used per treatment was around 10.

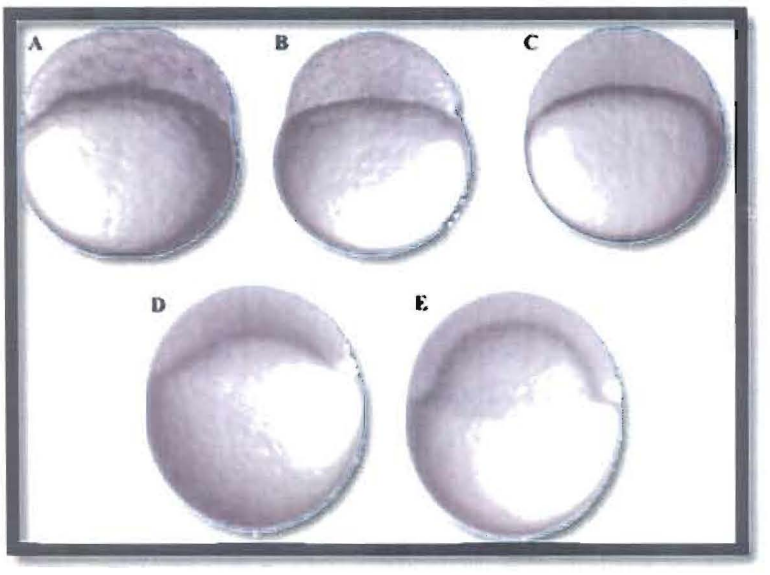


Fig 2.2: Zebrafish embryo development stages for analysis of Oct4 and Sall4 protein expression. A: Early Blastula stage, B: High stage, C: Oblong stage, D: Dome stage, E: 50% epiboly (Kimmel et al, 1995).

2.3 DEVELOPMENT OF PLURIPOTENCY MARKERS

One of the factors required for the successful establishment of zebrafish ES cell lines, is the detailed analysis of pluripotency genes in this specie. There is not much reported work on the pluripotency markers available in this specie (Robles et al, 2011). The aim of these experiments was to analyse the expression of human and mouse pluripotency genes i. e. Oct4 and Sall4 in different zebrafish embryo stages. Robles et al (2011) reported a peak expression of Pou and Sall4 at oblong stage embryos as compared to somatic cells. We aim here to analyze the protein expression employing immunocytochemistry.

Commercially there are no antibodies against these antigens are present specifically for zebrafish specie. For this purpose bioinformatic analysis was done to identify the homology in the antibody binding site in both species.

2.3.1 BIOINFORMATIC ANALYSIS

The protein sequences of Oct4 and Sall4 in zebrafish were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) (Table 2.1). The sequences were run against human and mouse sequences for Oct4 and Sall4 respectively in ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to identify the homology in antibody binding site (Fig 2.3 and 2.4). As the primary antibodies used in the experiments for the analysis of protein expression in zebrafish embryo stages, were specific for human (Oct4) and mouse (Sall4) species.

Table 2.1: Table shows gene name, gene ID and accession no. of mRNA and protein sequence for mouse, human and zebrafish.

Gene	Specie	Gene ID	Ref Seq (mRNA)	Ref Seq(Protein)
Oct4	*Mus musculus*	18999	NM_013633.1	NP_038661.1
	Homo sapiens	5460	NM_002701.3	NP_002692.2
	Danio rerio	303333	NM_131112	NP_571187
Sall4	*Mus musculus*	99377	NM_175303.3	NP_780512.2
	Homo sapiens	57167	NM_020436.3	NP_065169.1
	Danio rerio	572527	NM_001080609.1	NP_001074078.1

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gi|18859249|ref|NP_571187.1|     MTERAQSPTAADCRPYEVNRAMYPQAAGLDGLGGASLQFAHGMLQDPSLI 50
gi|400659|sp|Q01860.1|PO5F1_HU   MAGHLASDFAFSP----------PPGGGGDGPGGP----------EP--- 27
                                 *: :  * * .          * ..* ** **.         :*

gi|18859249|ref|NP_571187.1|     FNKAHFNGITPATAQTFFPFSGDFKTNDLQGGDFTQPKHWYPFAAPEFTG 100
gi|400659|sp|Q01860.1|PO5F1_HU   ------------------------------GWVDPRTWLSFQGPPGG- 44
                                                             .:.:*: * .* .*

gi|18859249|ref|NP_571187.1|     QVAGATAATQPANISPPIGETREQIKMPSEVKTEKDVEEYGNEENKPPSQ 150
gi|400659|sp|Q01860.1|PO5F1_HU   ----------PGIGPGVGPGSEVWGIP---PCPPPYEFCGGMAYCGP-- 78
                                           ..*.* :*   *   :*        *  *.     *

gi|18859249|ref|NP_571187.1|     YHLTAGTSSVPTGVNYYTPWNPNFWPGLSQITAQANISQAPPTPSASSPS 200
gi|400659|sp|Q01860.1|PO5F1_HU   ---QVGVGLVPQGG--LETSQP---EGEAGVGVESNSDGASPEPCTVTP- 119
                                     .*.. ** *     . :*    * : : .::* . *.* *.: :*

gi|18859249|ref|NP_571187.1|     LSPSPPGNGFGSPGFFSGGTAQNIPSAQAQSAPRSSGSSSGGCSDSEEEE 250
gi|400659|sp|Q01860.1|PO5F1_HU   -----------------G--AVKLEKEKLEQNPEES----------QDI 139
                                               *  * :: . : :. *..*          ::

gi|18859249|ref|NP_571187.1|     TLTTEDLEQFAKELKHKRITLGFTQADVGLALGNLYGKMFSQTTICRFEA 300
gi|400659|sp|Q01860.1|PO5F1_HU   KALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFGKVFSQTTICRFEA 189
                                 .   ::****** **:******:*******:** *:**:***********

gi|18859249|ref|NP_571187.1|     LQLSFKNMCKLKPLLQRWLNEAENSENPQDMYKIERVFVDTRKRKRRTSL 350
gi|400659|sp|Q01860.1|PO5F1_HU   LQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAE-TLVQARKRKR-TSI 237
                                 ***********:****:*::**:*.** *:: * * .:*::***** **:

gi|18859249|ref|NP_571187.1|     EGTVRSALESYFVKCPKPNTLEITHISDDLGLERDVVRVWFCNRRQKGKR 400
gi|400659|sp|Q01860.1|PO5F1_HU   ENRVRGNLENLFLQCPKPTLQQISHIAQQLGLEKDVVRVWFCNRRQKGKR 287
                                 *. **. **. *::****.  :*:**:::****:****************

gi|18859249|ref|NP_571187.1|     LALPFDD-ECVEAQ--YYEQSPPPPPHMGGTVLPGQGYPGPAHPGGAPAL 447
gi|400659|sp|Q01860.1|PO5F1_HU   SSSDYAQREDFEAAGSPFSGGPVSFPLAPGPHFGTPGYGSPHFTALYSSV 337
                                  :  : : * .**    :. .* . *   *. :   ** .* ...  .::

gi|18859249|ref|NP_571187.1|     YMPSLHRPDVFKNGLHPGLVGHLTS- 472
gi|400659|sp|Q01860.1|PO5F1_HU   PFP---EGEAFPPVSVTTLGSPMHSN 360
                                  :*   . :.*     . * . : *
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Fig 2.3: Sequence alignment of zebrafish POU against human POU5f1 (http://www.ncbi.nlm.nih.gov/). Antigenic peptide was raised from within residues 300 to the C-terminus of human Pou5f1

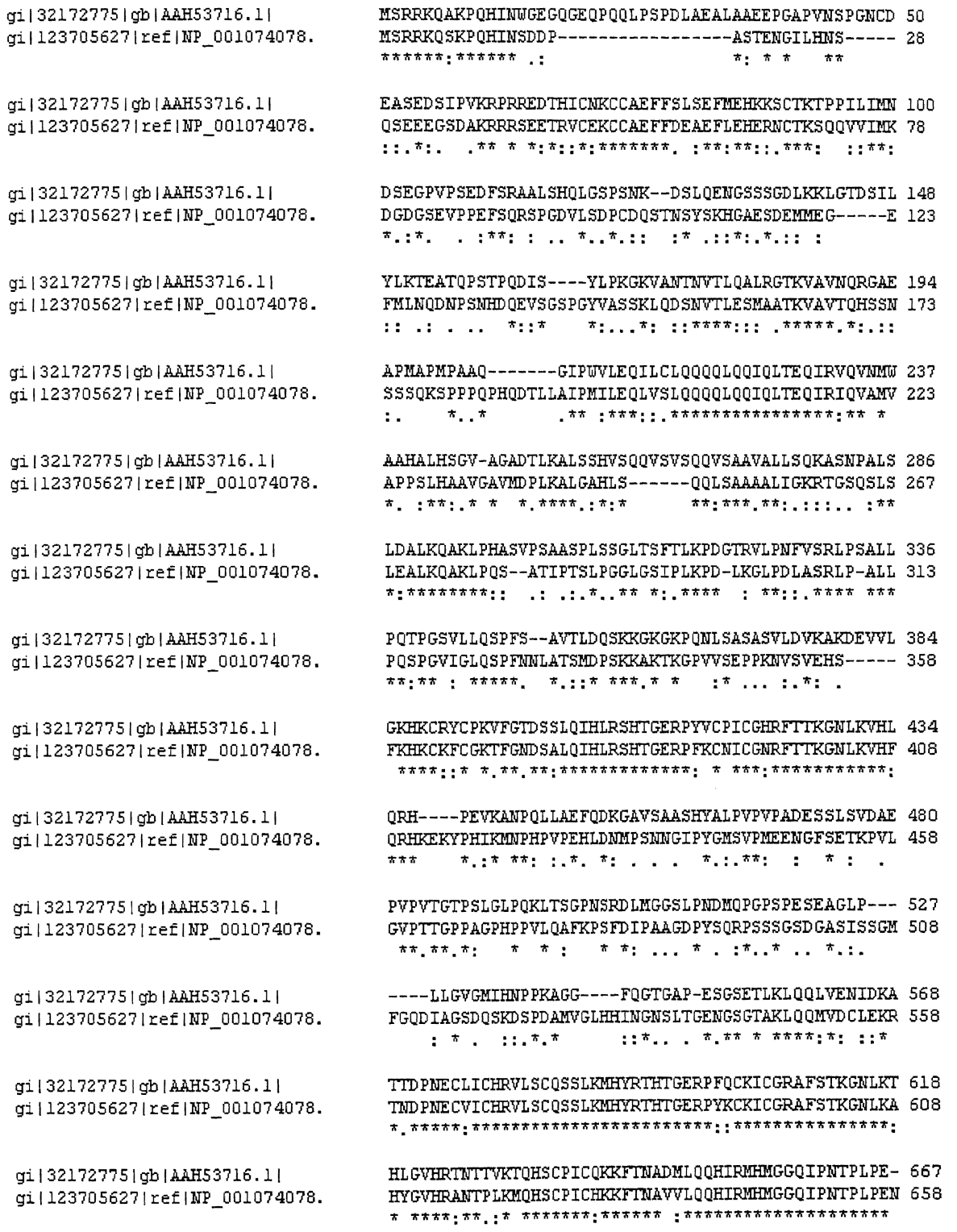


Fig 2.4: Sequence alignment of zebrafish Sall4 gene against mouse Sall4 (http://www.ncbi.nlm.nih.gov/). Antigenic peptide was raised from within residues 350 to 450 of mouse Sall4.

2.3.2 IMMUNO CYTOCHEMISTRY (ICC)

To determine the localization of proteins in cultured cell, tissues, oocytes and embryos, ICC employs antibodies raised against specific proteins of interest. For the successful detection of antigen samples need to be fixed, which helps in preserving the antigenicity of proteins. The sample also needs to be permeabilized if protein of interest is not on the cell surface. Often antibodies can bind non-specifically to proteins other than they are raised against. For this purpose samples are pre-incubated in a blocking solution which contains BSA that bind to these non-specific sites. Consequently the primary antibodies bind to the higher affinity specific sites than the blocking proteins. In order to remove excess antibody that has not bound to the target protein samples need to be washed after primary antibody incubation. Cells are than incubated with secondary antibody that is raised against primary antibody and contains a fluorescent tag. After further washes to remove excess secondary antibody, samples are ready to be visualized for the localization of target protein within a cell with fluorescent microscopy.

Confocal Microscope is a high powered imaging device that image either by reflecting light off the specimen or stimulating fluorescence from dyes applied to the specimen. It allows visualization of fluorescence along one plane, providing detailed analysis of protein localization in cells. As compared to conventional microscope it creates sharper, more detailed 2D images and allows collection of data in three dimensions (Semwogerere and Weeks, 2005)

2.3.2.1PROCEDURE

2.3.2.1.1EMBRYO COLLECTION

Embryos were collected at different developmental stages e.g. early blastula stage, high stage, oblong stage, dome stage and 50% epiboly.

After collection, embryos were placed in pronase solution (0.5 mg/ml) for 10 min or until chorions break apart. Dechorionated embryos were placed in fresh embryo medium. Cells were dissociated by gentle pipetting. Dissociated cells were placed on gelatin coated coverslips and left for attachment.

2.3.2.1.2 IMMUNOCYTOCHEMISTRY

The chemicals used in immunocytochemical analysis are shown in Table 2.2. Coverslips were fixed in 4% formaldeyhde in PBS for 20 minutes, washed three times with PBS and then permeabilized in 0.5% triton X-100 in PBS for 10 minutes at room temperature. Cover slips were then washed in 0.1% triton X 100 in PBS for three times and blocked in 2% BSA in 0.1% triton X 100 at 4° C overnight.

Blocked coverslips were incubated with primary antibody, rabbit polyclonal to Oct 4 and rabbit polyclonal to sall4 (Abcam, UK) overnight at 4°C. After three washes with PBS, coverslips were incubated with secondary antibody Alexa flour 594 and 488 anti- rabbit IgG (Invitrogen, UK) for 2 hours at room temperature. Table 2.3 shows the detail of antibodies and their concentrations. As negative controls, cells were incubated only with secondary antibody. After washing three times with PBS, cover slips were mounted on slides in vecta shield containing 4', 6-diamidino-2-phenylindole (DAPI).

Table 2.2 List of chemicals prepared for immunocytochemical analysis

CHEMICAL S	USE
4% formaldehyde in PBS	Fixing solution.
0.5% triton X-100 in PBS	Permeabilizing solution.
0.1% triton X-100 in PBS	Washing solution.
2% BSA with 0.1% triton X-100	Blocking solution.
Phosphate buffer saline (PBS)	Washing solution.

Table 2.3 Antibody details and concentration

Protein	Primary AB	Primary AB concentration	Secondary AB	Secondary AB concentration
Oct4	Rabbit IgG anti-Oct4 (Abcam)	5 µg/ml	Alexa Flour ® 594 anti-rabbit IgG	5 µg/ml
Sall4	Rabbit IgG anti-Sall4 (Abcam)	5 µg/ml	Alexa Flour ® 488 anti-rabbit IgG	5 µg/ml

All the experiments were run in triplicates.

2.3.2.1.3 CONFOCAL MICROSCOPY

Immunostained samples were examined and imaged upside down using the Leica TCS SP5 confocal microscope and scanner and fluorescence data was collected using Leica Confocal software (Leica). DAPI fluorescence was excited at 405nm, FITC (Fluorescein isothiocyanate) was excited at 488nm and TRITC (Tetramethyl Rhodamine Iso-Thiocyanate) was excited at 594nm. Each image was produced from the average fluorescence data obtained from 4 scans, thereby reducing background fluorescence.

2.3.2.1.4 QUANTIFICATION OF PROTEIN EXPRESSION

The protein expression in the immunostained cells was assessed on the basis of the size of protein clumps, distances between the clumps and their distribution in the cell or nucleus. Three different areas of the coverslip were analyzed per replicate. All the measurements were carried out using Image J software.

2.4 CULTURING OF ZEBBRAFISH BLASTOMERES

2.4.1 MEDIA PREPARATION

Embryonic cells were derived in LDF basal nutrient medium. LDF media was prepared by combining Leibowitz's L-15, Dulbecco's Modified Eagle's Medium and Ham's F12 in a ratio of 50:35:15 supplementing with sodium bicarbonate (0.180g/l) and sodium selenite (10^{-8} M). The following culture media (Table 2.4) were available from sigma Aldrich: Leibowitz's L15, Ham's F 12, and Dulbecco's modified eagle's (DMEM). One liter of each medium was prepared by dissolving the powder in ddH2O. HEPES buffer was added at a final concentration of 15mM pH 7.2. The following antibiotics were used at penicillin and streptomycin sulfate at a final concentration of 30ug/ml and 50ug/ml respectively. The medium was filter sterilized before use.

Table 2.4 Chemical composition of culture medium

INGREDIENTS	CONCENTRATION	SOURCE
Leibowitz's L-15	50%	SIGMA- ALDRICH
Dulbecco's Modified Eagle's medium (DMEM)	35%	SIGMA- ALDRICH
Ham's F 12	15%	SIGMA- ALDRICH
Sodium bicarbonate	0.18g/l	SIGMA- ALDRICH
HEPES buffer	15mM	SIGMA- ALDRICH
Sodium selenite	10^{-8} M	SIGMA- ALDRICH
Penicillin	30µg/ml	SIGMA- ALDRICH
Streptomycin	50µg/ml	SIGMA- ALDRICH

2.4.2 GROWTH FACTORS

The information on growth factors in the medium used in the experiments is provided in Table 2.5.

Table 2.5 List of growth factors supplemented in culture medium

Ingredients	Concentration	Source
Fetal Bovine Serum	5%	SIGMA-ALDRICH
Epidermal Growth Factor	50ng/ml	SIGMA-ALDRICH
Human Insulin	10μg/ml	SIGMA-ALDRICH

Human insulin the stock solution was prepared at 1 mg/ml in 20mM Hcl (final conc. 10ng/ml).

Epidermal growth factor the stock solution was prepared in1mg/ml in LDF medium (final conc. 50ng/ml).

Basic Fibroblast growth factor the stock solution was prepared at 25μg/ml in filtered 20mM Tris (pH 7).

2.4.3 GROWTH EVALUATION

Cell growth was evaluated on the basis of two parameters i.e. measurement of diameter of embryonic cell colonies and colony no. The calculations were done for three different areas of the well per replicate. The diameter was measured using Image J software.

2.5 STATISTICAL ANALYSIS

Results were expressed as mean ± SD. Student t test was used to evaluate the statistical significant difference between the groups ($p< 0.05$), means, standard deviation and t test were calculated using Excel version 2010 for windows.

2.6 IMAGE ANALYSIS

All image analysis on cell cultures and immunostained samples was done by Image J software (http://rsbweb.nih.gov/ij/).

3 RESULTS AND DISCUSSIONS

3.1 Identify molecular markers for pluripotency ES cells in zebrafish

The establishment of mouse embryonic stem cell culture has enabled the production of knock out mutants in this specie which is a valuable approach to study gene function (Van Der Weyden et al, 2002). There has been a continuous effort for establishing of ES cell lines in other species as well. Although the methods for zebrafish ES cell like derivation has been described in literature, efficient production of gene knock outs has not been achieved in this specie (Robles et al, 2011). Targeted inhibition of gene expression in zebrafish using antisense phosphoramidite morpholino oligomers (MOs) has been studied (Nasevicius and Ekker, 2000) and both Doyon et al (2008) and Meng et al (2008) demonstrated the successful use of zinc finger nucleases for targeted gene inactivation in zebrafish. However cell mediated gene transfer in ZF cell cultures is still not attained. There are reports demonstrating the germ line competency of zebrafish cell cultures (Fan et al, 2004) and homologous recombination has been carried out in these cultures (Fan et al, 2006). Regardless of these advances, only blastula derived cell lines have been established instead of ESC lines (Xing et al, 2008). One of the major hurdles is the lack of knowledge of pluripotency markers in this specie. Studies were carried out by Robles et al (2011) on expression of different pluripotency genes in zebrafish embryonic stages and cultures.

The aim of this study was to investigate the expression of two pluripotency markers Oct4 and Sall4 in different zebrafish embryonic stages e.g. from early blastula to high, oblong, dome and 50% epiboly stage. It is reported (Kimmel and Warga, 1986; Ho, 1992; Ho and Kimmel, 1993; Strehlow and Gilbert, 1993) that pluripotent cells can be successfully isolated from blastula stages in zebrafish. We aimed to identify most suitable blastula stage in zebrafish for the isolation of pluripotent cells for successful establishment of embryonic stem cell cultures.

3.1.1 OCT4

Oct4 is an important and well recognized pluripotency marker for human and mouse embryonic stem cells (Nichols et al, 1998; Niwa et al, 2000). In the present study, investigations were carried out on Oct4 protein expression in zebrafish at different embryo developmental stages.

In order to see the localization and abundance of Oct4 protein in different embryo developmental stages; cells from all embryo stages to be analysed were isolated, fixed, permeabilized and blocked as described in section 2.3.2.1.2. The blocked cells were then incubated in primary antibodies overnight and with secondary antibody for 2 hours with the concentrations stated in Table 2.3. Confocal microscopy was carried out as described in section 2.3.2.1.3.

The immunostained cells revealed different staining patterns for protein expression. The protein expression was categorized on the basis of the size of the protein clump, its arrangement in the nucleus and intensity of expression (the area of nucleus covered with protein) and cytoplasmic staining. There were broadly four categories (Fig 3.1) observed on the basis of criterion mentioned above and observed the type of pattern present at each stage.

In early blastula stages there was cytoplasmic staining evident in addition to nuclear localization. Cytoplasmic staining was also reported in murine pre-implantation embryos at around 8 to 16-cell stage and then protein started to localize in nuclei (Palmieri et al, 1994; Hubner et al, 2003). The protein speck size was roughly around 0.1μm and was sparsely present throughout the nucleus. The cytoplasmic staining indicates that Oct4 protein is being synthesized at this stage and is also being translocated to the nucleus. The next category showed the peak expression of Oct4 protein. Big sizes of protein clumps around 2μm were compactly present throughout the nucleus. The protein expressed approximately the whole area of nucleus. There was no Oct4 cytoplasmic staining found and it can be assumed that after early blastula stage the Oct4 gene is switched off and all the protein has localized in nucleus to perform its role to maintain pluripotency. This staining pattern was assumed to be the highest protein expression and can be

suggested that pluripotent cells should show protein expression similar to this category. In our analysed stages this expression pattern was observed in only high stage blastomeres. The third category showed low levels of Oct4 protein as compared to category 2. The protein clump size was reduced to app. 1 μm and the area of nucleus covered by the protein was also reduced. However the clumps were present interconnected throughout the nucleus. In fourth category the protein speck size was further reduced to around 0.05 μm and protein specks were scattered though out the nucleus and were apart from each other. In the oblong, dome and 50% epiboly blastomeres, two types of staining categories i.e. Category 3 and 4 were observed. Different ratio of cells from the stages was present in both categories. The results indicated that blastomeres are most pluripotent at high stage and started to lose pluripotency afterwards as indicated by the decrease in protein expression.

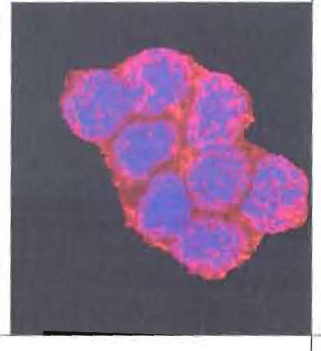
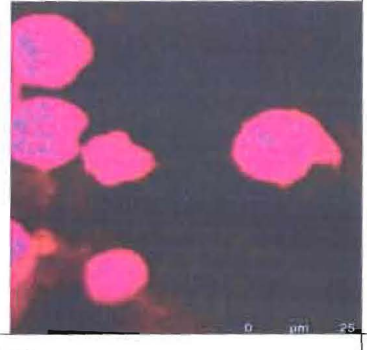
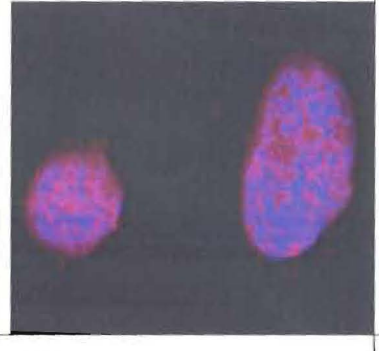
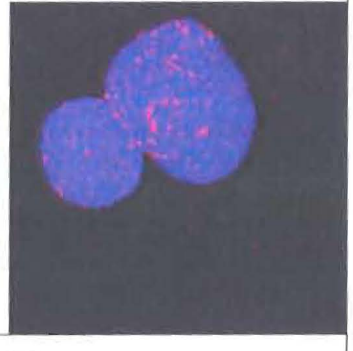
EMBRYO STAGE	CATEGORIES			
	Protein specks (≤0.1μm), interconnected throughout nucleus.	Protein clumps (>2μm) compactly present throughout nucleus.	Protein clumps app. (1μm), interconnected throughout nucleus.	Protein specks (<0.05μm) present throughout nucleus with 1 um apart.
Pictorial presentation				
Early	100%	0%	0%	0%
High Stage	0%	76%	23%	0%
Oblong	0%	0%	65%	34%
Dome Stage	0%	0%	86%	13%
50% epiboly	0%	0%	36%	63%

Fig 3.1: Different Staining patterns observed for Oct4 protein localization. Cytoplasmic and nuclear localization of Oct4 at early blastula stage. Oct4 protein started to localize in nucleus after early blastula stage with a dense expression at High stage. At later stages Oblong, Dome and 50% epiboly the protein expression has decreased.

Studies carried out in human and murine embryonic stem cells demonstrated the crucial role played by Sall4 in the maintenance of pluripotency. Sall4 plays its role through transcriptionally modulation of Oct4 and Nanog in maintaining undifferentiated phenotype of ESCs and governing the fate of ICM (Yang et al, 2008). Down-regulation of Sall4 resulted in subsequent low levels of Oct4, Sox2, Klf4 and c-Myc; these genes are involved in reprogramming of somatic cells to IPSCs. So Sall4 can be proposed as a key regulator in reprogramming process (Yang et al, 2008). There is limited information in the literature on the role of Sall4 in zebrafish embryos and cultured embryonic cells. Robles et al (2011) reported the expression of Sall4 gene in zebrafish oblong stage embryos in comparison with adult somatic cells.

Sall4 protein expression at different embryo development stages were investigated using immunostaining. As observed with Oct4 expression, Sall4 protein also showed various expression patterns in the cells. These patterns were classified as shown in Fig 3.2. The different developmental stages were than classified into the categories. Staining patterns were broadly classified into 4 categories on the basis of size of the protein, their spatial location in nucleus, and cytoplasmic localization.

The protein staining patterns were somewhat similar to Oct4 expression and it confirms that both genes are interrelated. Category 1 consists of expression of Sall4 protein localization to cytoplasm and protein speck size was around 0.1 μm present interconnected throughout the nucleus. Early blastula stage blastomeres showed this type of staining pattern. Similar to Oct4 expression at early blastula stage, there was cytoplasmic expression of Sall4 evident which indicates that Sall4 protein is being synthesized at this stage. However as we moved to later stages no cytoplasmic expression of Sall4 protein was found. It can be supposed that Sall4 gene has turned off. Category 2 comprised of staining pattern exhibiting protein clumps greater than 1 μm which are compactly present throughout the nucleus. High levels of Sall4 protein were expressed as evident from the fully immunostained nucleus. The third category consisted of protein clumps which have reduced in size to less than 1μm and present throughout the nucleus.

However the Sall4 levels are lower as compared to category 2. The fourth category constitute of protein specks around 0.05μm present throughout the nucleus with 1μm apart. High stage blastomeres were placed in Category 2 and 100% of the blastomeres showed this protein expression and it is assumed to be the highly expressed Sall4 protein category. All of the oblong stage blastomeres showed staining pattern of Category 3 in which protein expression levels are reduced as compared to Category 2. As we move on to the next blastula stage i.e. dome stage, it showed two type of staining patterns. A greater proportion of the cells showed staining pattern similar to Category 3 and a lesser no. showed of Category 4. At 50% epiboly all of the cells showed protein expression pattern of Category 4. This indicates that maximum protein expression is at high stage and consequently it can be proposed that cells are most pluripotent at this stage. In later stages the protein expression is decreased evident from small protein size and it can be proposed that cells have started to lose pluripotent nature.

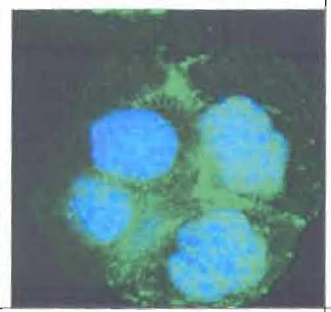
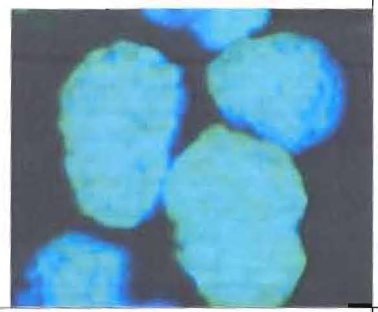
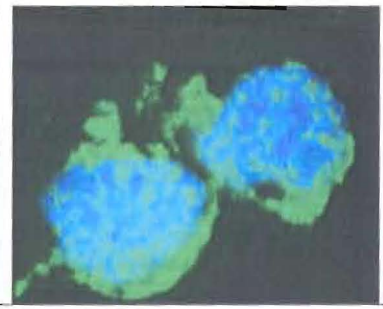
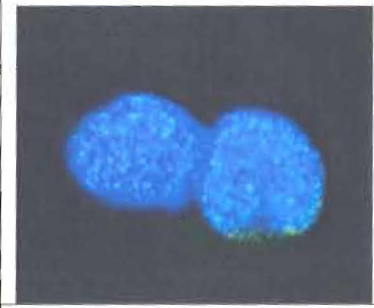
EMBRYO STAGE	CATEGORIES			
	Protein specks (0.1μm) interconnected throughout nucleus.	Protein clumps (>1μm) compactly present throughout nucleus.	Protein specks <1μm present throughout nucleus.	Protein specks (<0.05μm) present throughout nucleus with 1 um apart.
Pictorial presentation				
Early Blastula	100%	0%	0%	0%
High Stage	0%	100%	0%	0%
Oblong	0%	0%	100%	0%
Dome Stage	0%	0%	80%	5%
50% epiboly	0%	0%	0%	100%

Fig 3.2: Different staining patterns observed for Sall4 protein localization. Cytoplasmic and nuclear localization of Sall4 protein at early blastula stage. Sall4 protein started to localize in nucleus after early blastula stage with a dense expression at High stage. At later stages Oblong, Dome and 50% epiboly the protein expression has decreased.

3.2 EMBRYONIC CELLS DERIVATION FROM ZEBRAFISH HIGH STAGE EMBRYOS

Studies carried out on the pluripotency markers Oct4 and Sall4 at different zebrafish embryo stages demonstrated highest expression of pluripotency genes at high stage (Fig 3.3). At High stage cells are considered to be pluripotent as it is widely accepted that cell type determination does not occur before the beginning of gastrulation. Lin et al (1992) demonstrated that a small no of cells from blastula stage when transplanted into other blastula stage embryos participated in formation of many cell types including a germ line in the recipient. So, the blastomeres were isolated from this stage and derived in LDF medium supplemented with epidermal growth factor, bovine insulin and FBS (5%), on gelatine coated wells.

There have been reported methods for the derivation of zebrafish ES cells (Fan et al, 2004; Xing et al, 2008). However the medium composition is quite complex and consists of various supplements e.g. zebrafish embryo extract, trout plasma which are not available at ease in every laboratory. We aimed to simplify the protocol for culturing of zebrafish embryonic cells.

Fig 3.3: Zebrafish embryo at high stage (Kimmel et al, 1995)

Although methods for ES cell derivation from zebrafish embryo have been described in the literature these cultures are not suitable for cell mediated gene transfer in this specie. The most suitable stage identified for stem cell derivation is oblong stage but the cultures obtained are blastula derived cell lines instead of ES

cell lines (Xing et al, 2008). We have attempted to isolate pluripotent cells from zebrafish embryos at high stage based on our immunocytochemistry analysis of the expression of pluripotency genes.

3.2.1 EFFECT OF FOETAL BOVINE SERUM (FBS) ON EMBRYONIC CELL CULTURE

Blastomeres were isolated from high stage zebrafish embryos and cultured on gelatine coated 24 well plate. 10 embryos per condition were used. Zebrafish embryonic cells were cultured in LDF medium supplemented with epidermal growth factor (50ng/ml), bovine insulin (10µg/ml) and foetal Bovine serum (5%).

The cell growth was evaluated by measuring the diameter of embryonic cell colonies and the no. of colonies/ mm^2 present as described in section 2.4.3. The cell growth appeared to be slow as there was not a considerable No. of colonies and the increase in their diameters over time. So another FBS concentration (10%) was tested.

Both parameters i.e. embryonic cell colony diameter and no of colonies were determined in both FBS concentrations. Fig 3.4 and Fig 3.5 show the comparison of 5% FBS and 10% FBS conditioned medium using either of the parameters. There were significant differences ($p<0.05$) between **5% and 10% FBS supplemented LDF medium** when either of the parameters was used.

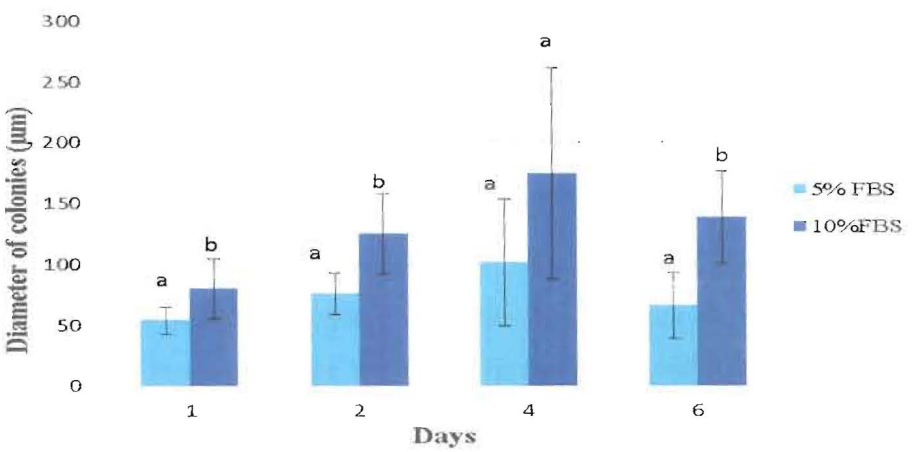


Fig 3.4: Effect of FBS on diameter of embryonic cell colonies in 5% and 10% FBS supplemented LDF medium. Error bars represent standard deviation from mean. Different letters indicate significant difference ($p<0.05$, $n=9$) between the two conditions on same day.

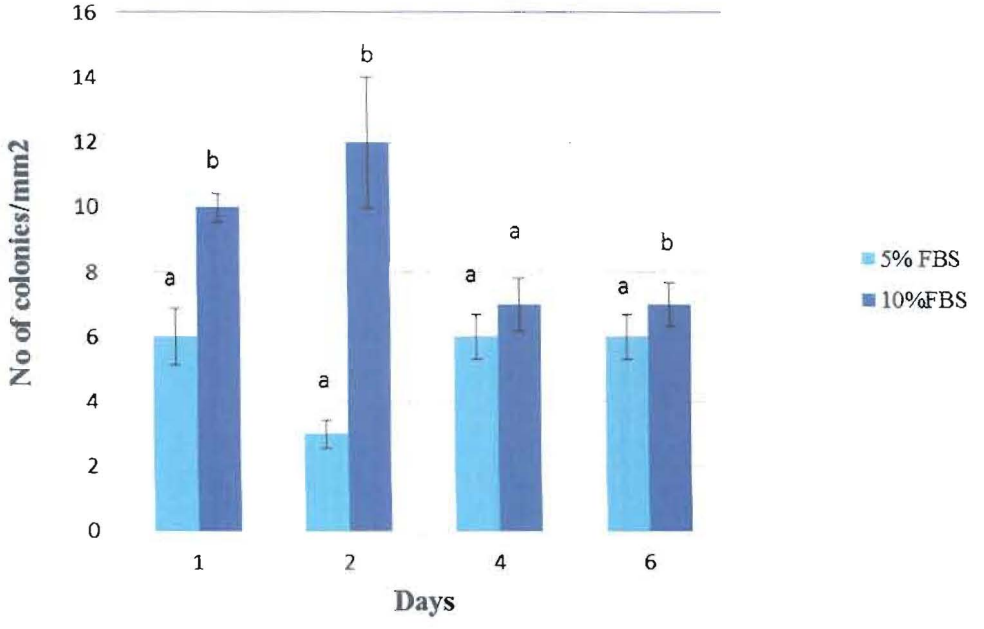


Fig 3.5: Effect of FBS on embryonic cell colony no. in 5% and 10% FBS supplemented LDF medium. Standard bars indicate standard deviation from the mean. Different letters indicate significant difference between different culture conditions on the same day ($p<0.05$, $n=9$).

These results indicated that 10% FBS supported better growth of embryonic cell colonies. Fig 3.6 shows the appearance of embryonic cell colonies in 10% FBS conditioned medium. Cultured embryo cells showed aggregate formation on Day 1 (Fig 3.4 A). Appearance of embryonic cell colony on Day 3 showed an increase in aggregate size. Some colonies exhibited neurite formation (projections extending from the periphery of aggregates (Fig 3.4 B). Fig 3.4 D displays the appearance of melanocyte (red arrow) in embryonic cell colony and differentiation of few embryonic stem cells in embryo fibroblasts (black arrow).

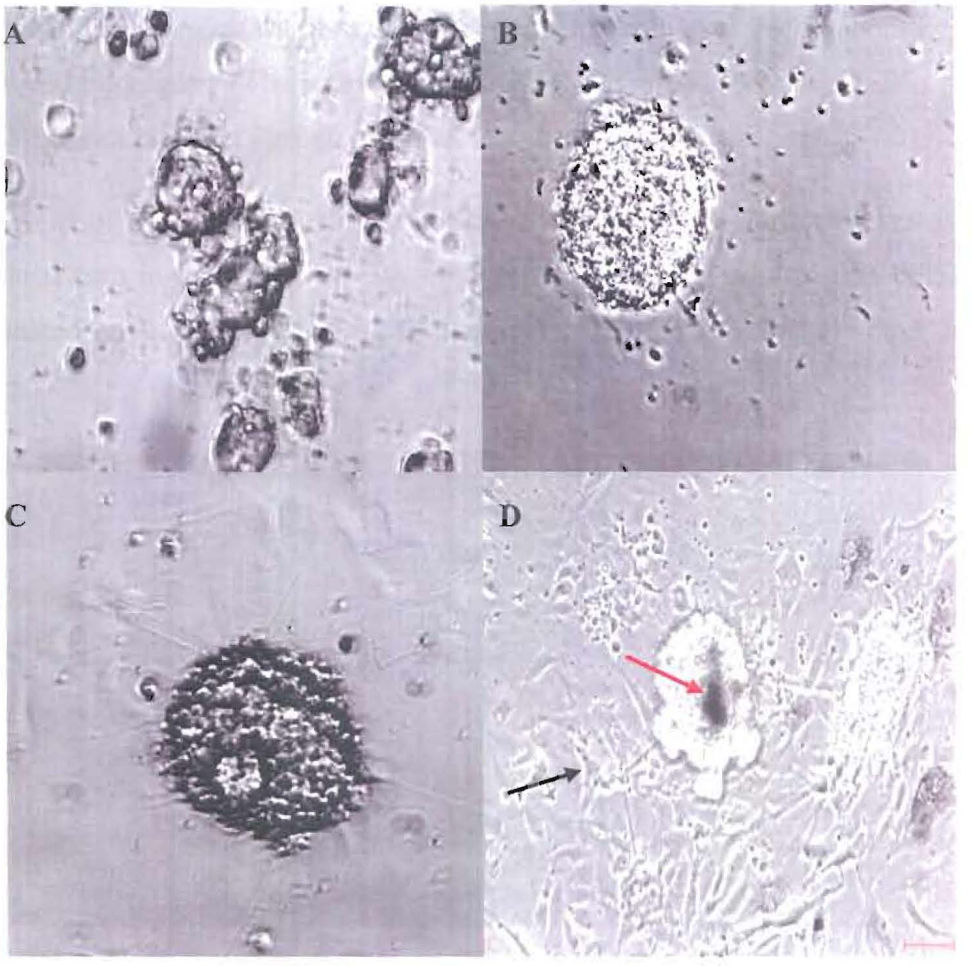


Fig 3.6: Phase contrast photomicrograph of embryonic cell colony. (A) Culture (Day 1) embryo cell aggregates (B) Culture (Day3) without any neurite formation (C) Culture (Day 6) embryonic cell colony with projections (neurite) extending out of cluster (D) culture (Day 6) the differentiation of few embryonic cells into embryo fibroblast cells (black arrow) and presence of mealnocytes (red arrow).

3.2.2 EFFECT OF FIBROBLAST GROWTH FACTOR (bFGF) ON CELL CULTURE

In our culture system, blastomeres isolated from high stage were cultured in LDF medium supplemented with epidermal growth Factor and Bovine insulin. The isolated cells were of spheroid shape when cultured. The cells approached each other and tend to form cell colonies. These colonies were circular in morphology and tend to grow as evidenced from their increase in diameter. However approximately on Day 3 there was appearance of outgrowths (long extension)

shown in Fig 3.7 B, which was assumed to be neurites (Sun et al, 1995), emerging from the cell aggregates. The neurite formation was assumed to be indication of neuronal differentiation in the cell colonies.

The addition of bFGF to the culture medium resulted in inhibition of neurite formation shown in Fig 3.7 A till day 6 of culturing as compared to non-bFGF supplemented medium where neurite formation started on approximately day3 of culturing.

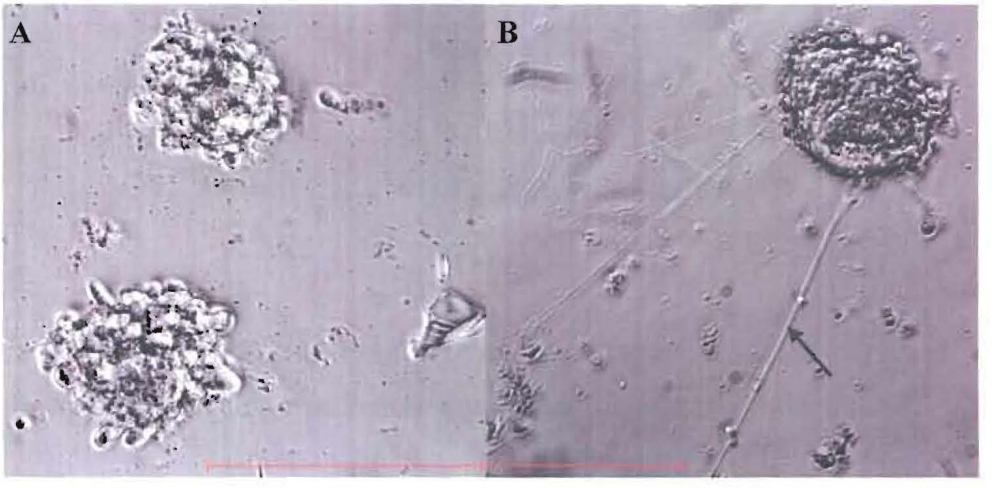


Fig 3.7: Phase contrast photomicrographs of zebrafish embryonic cell colony at Day 6. A: Embryonic cell colonies in bFGF supplemented medium. There was no evident growth of neurite (projections) from cell clusters. B: Embryonic cell colony in non-bFGF supplemented medium with neurite (arrow), indicating neural cell differentiation has occurred, extending out of the aggregates (scale bar 250 μm).

It has been reported previously that addition of bFGF to the zebrafish embryonic cultures resulted in stimulation of proliferation and melanogenesis inhibition in culture system (Bradford et al, 1994). Singh et al (2001) demonstrated that addition of bFGF to zebrafish early embryo cultures resulted in the suppression of neural-specific markers. Likewise there are evidence that bFGF in concert with other growth factors play role in the maintenance of undifferentiated hESC in absence of feeder layers (Wang et al, 2005; Xu et al, 2005; Xu et al, 2005). We report here that addition of bFGF in our cultures resulted in total inhibition of neurites formation in cell colonies.

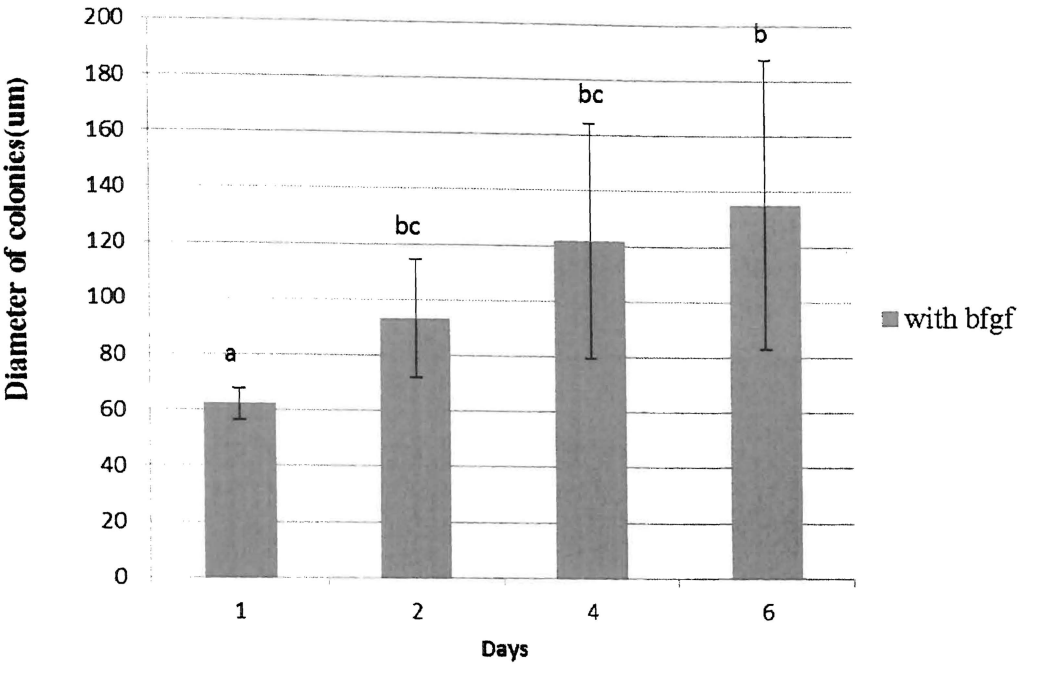


Fig 3.8: Diameter of Embryonic colonies cultured in bFGF supplemented medium. Different letter indicates significant difference ($p<0.05$, n=9).

Fig 3.8 shows the growth of zebrafish embryonic cell colonies in bFGF supplemented medium. There was increase in diameter of embryonic cell colonies over the period of six days. Therefore, bFGF had no effect in inhibiting the growth of colonies.

Comparison of neurite forming colonies in culture medium without bFGF and with bFGF is shown in Fig 3.9 till day 6 of cell culturing. There was significant difference ($p<0.05$) found in the No. of neurite forming colonies in both conditions which shows that bFGF played an important role in inhibiting neuronal differentiation in embryonic cell colonies.

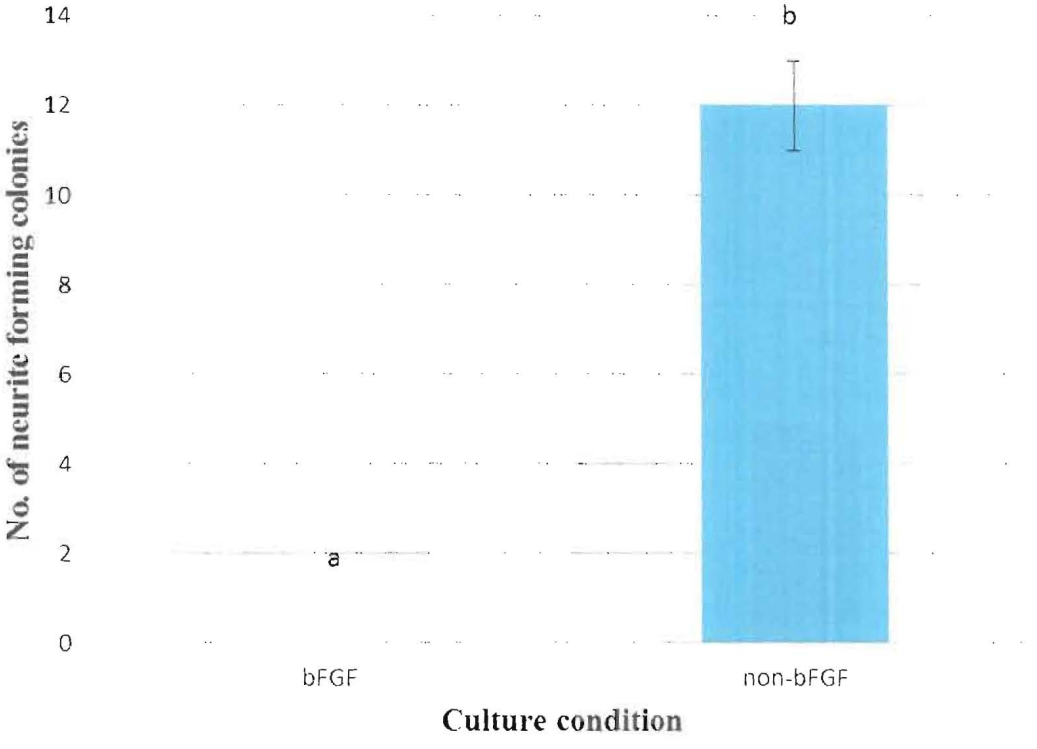


Fig 3.9: Comparison of neurite forming colonies in bFGF supplemented and non-bFGF supplemented medium till Day 6 of cell culturing. Different letters indicate significant difference ($p<0.05$, n=9).

Comparison of diameter and No. of embryonic cell colonies cultured in bFGF supplemented medium and non-bFGF supplemented medium is shown in Fig 3.10 and 3.11. There were no significant differences ($p<0.05$) found for both parameters i.e. embryonic cell colony diameter and number in both culture conditions. So bFGF neither enhanced nor inhibited the growth of embryonic cell colonies.

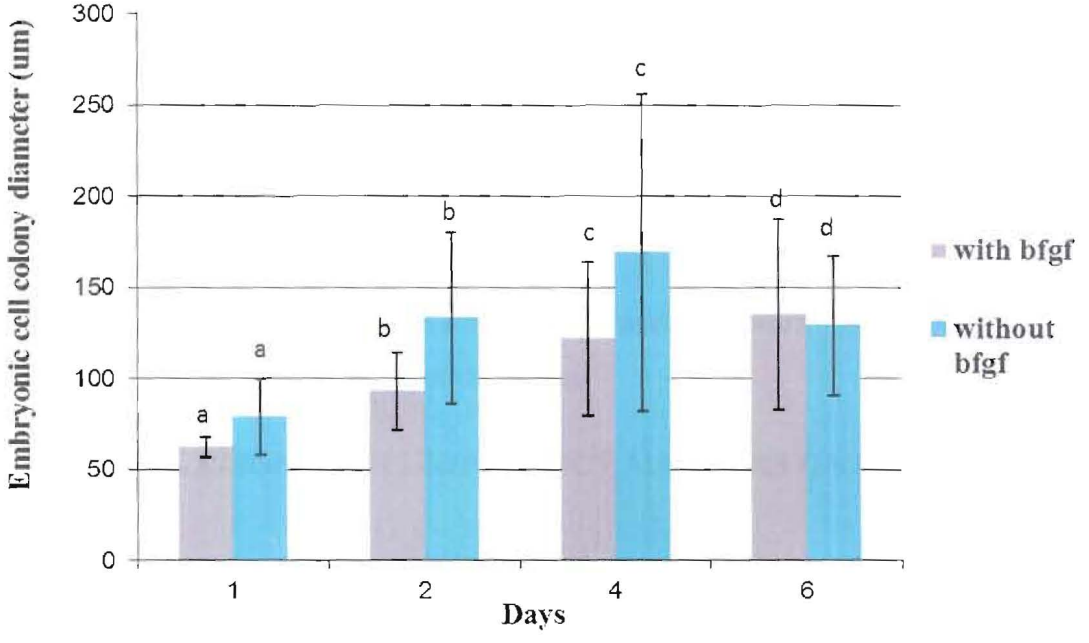


Fig 3.10: Comparison of embryonic cell colony diameter over the period of 6 days in bFGF supplemented and non-supplemented medium. Standard bars represent standard deviation.

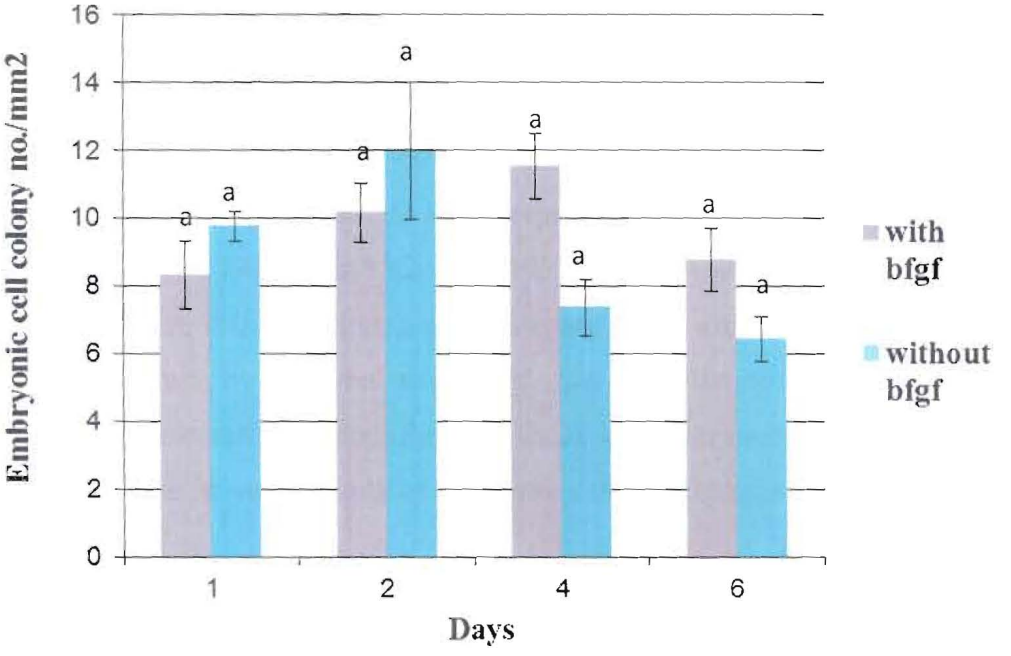


Fig 3.11: No. of embryonic cell colony in both medium conditions. Error bars represent standard deviation from the mean.

3.3 APPLICATION OF PLURIPOTENCY MARKERS ON CULTURED CELLS

The immunocytochemistry analysis of Oct4 and Sall4 protein in different zebrafish embryo stages revealed some promising results that these genes can be used as markers for the identification of pluripotent nature of zebrafish embryonic stem cells.

The aim of this experiment was to test the application of these markers on the embryonic cell cultures at various culture periods. The expression of Oct4 and Sall4 was analysed in embryonic cell colonies cultured in non-bFGf supplemented medium. In Fig 3.12 A the embryonic cell colony is stained with Oct4 on Day 6. The protein expression pattern was similar to Category 3 in which protein speck size has reduced to less than 1μm and though it is present throughout the nucleus but the expression is not very dense as compared to Category 2 expression which is assumed to be shown by the pluripotent cells. So it can be proposed that the cell colony has already started to lose pluripotency and it is also evident from the

culturing results that usually around Day 3 embryonic cell colonies started to differentiate. The other two images Fig 3.12 A & B are the immunostained zebrafish embryo fibroblasts. Their expression pattern was found to be similar to Category 4 which was exhibited by some cells at 50% epiboly stage and at this stage it is also considered that cell fate determination has occurred (Kimmel and Warga, 1986; Ho, 1992; Ho and Kimmel, 1993; Strehlow and Gilbert, 1993). The protein speck size was the minimum in Category 4 and can be considered as the expression shown by differentiated cells. So, the fibroblasts exhibited the expression pattern demonstrating that they have lost pluripotency. This validates that Oct4 marker is well capable of identifying the pluripotent status of cultured embryonic cells.

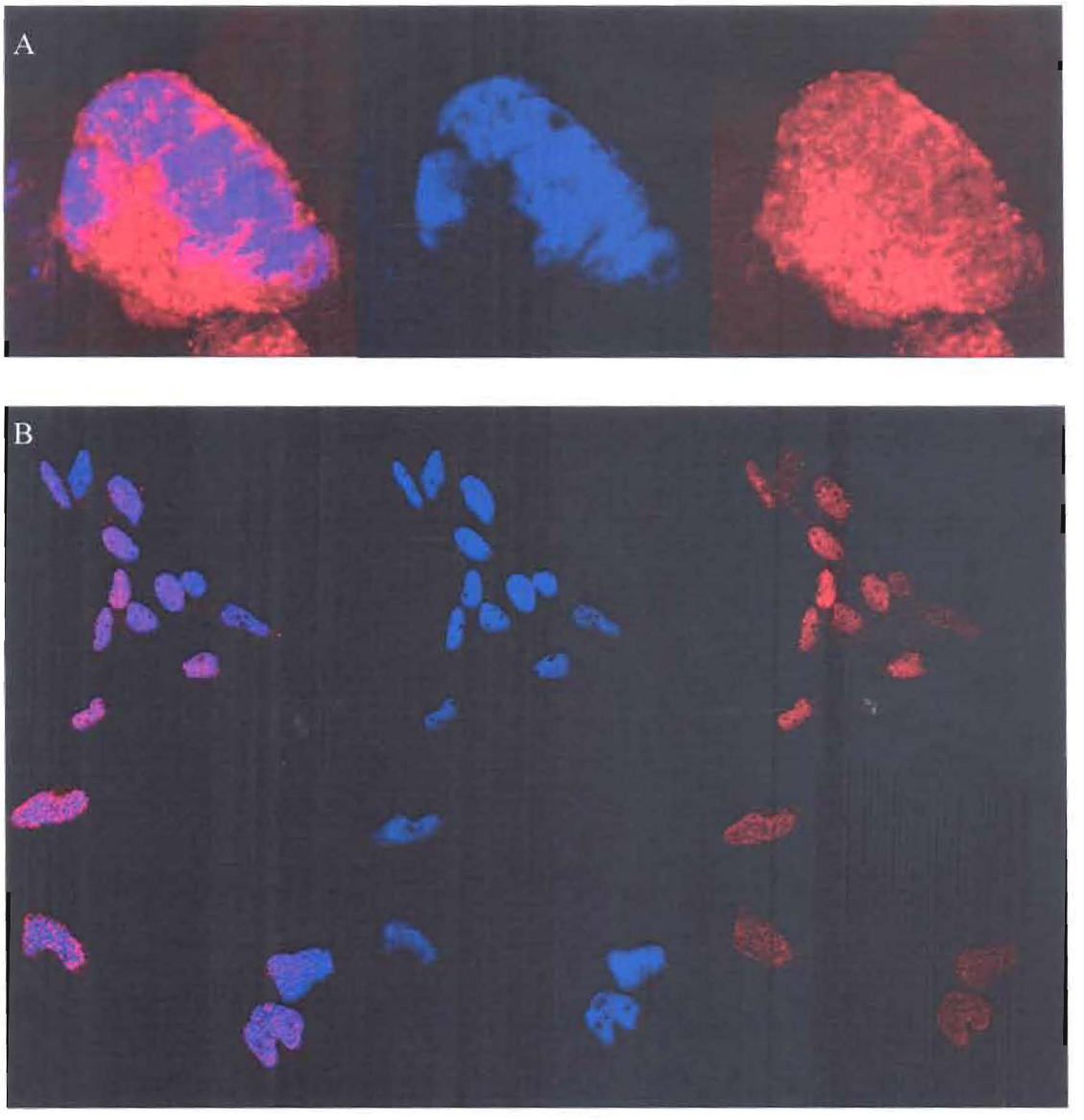


Fig 3.12: Immunocytochemistry analysis for Oct4 protein in embryonic cell cultures. (Merged image; DAPI, blue; Oct4 red) (A) Embryonic cell colony on Day 6. (B) Embryo fibroblasts (elliptical nuclei), on Day3 and Day4 in 10% FBS conditioned medium.

In Fig 3.13 the embryonic cell cultures are stained with Sall4 antibody. The two embryonic cell colonies stained on same Day 11 of culturing. The embryonic cell colony in Fig 3.13 A shows expression pattern similar to Category 3 in which protein size is reduced to <1μm and protein is present interconnected throughout the nucleus. The embryonic cell colony in Fig 3.13 B depicts the Sall4 protein expression of Category 4 in which the expression was reduced as compared to Category 2 which showed highest nuclear expression. The protein speck was reduced to less than 0.05 μm and were scattered in whole nucleus. The expression patterns depicted by embryonic cell colonies demonstrated that these colonies have already started to differentiate. This is in line with our cell culture results that the cell colonies showed signs of differentiation i.e. neurite, onwards Day 3 of culture in non-bFGF supplemented medium.

The third image Fig 3.13 C is of embryo fibroblast which showed low levels of Sall4 protein and also exhibited cytoplasmic staining. These results indicated that the Sall4 marker worked well for the cultured embryonic cells and is able to help in identification of differentiated or undifferentiated phenotype of embryonic cell colonies.

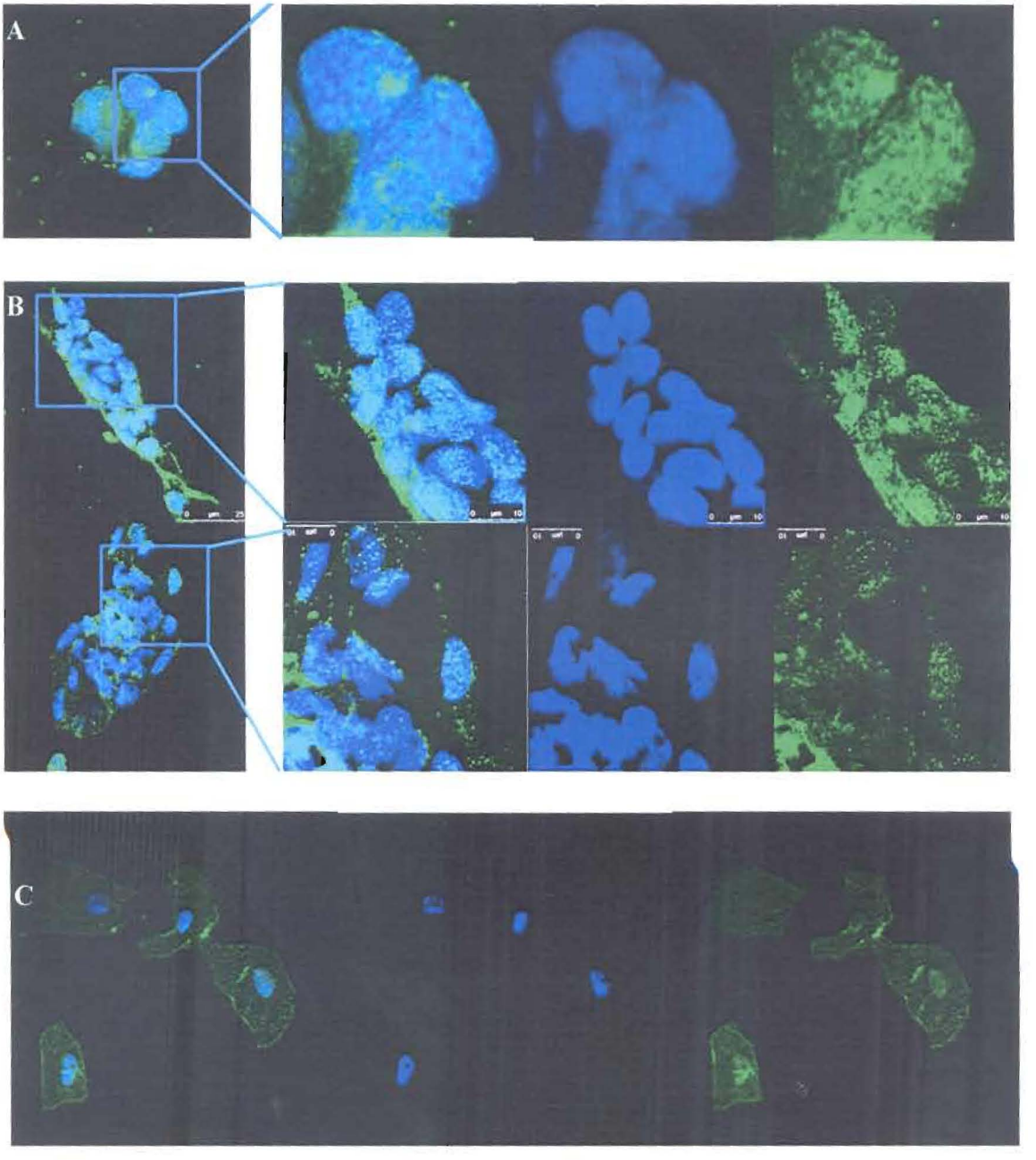


Fig 3.13: Immunocytochemistry analysis for the presence of Sall4 protein in embryonic cell cultures on Day 11 (Merged image; DAPI, blue; Sall4, green). (A & B) Sall4 protein expression pattern in embryonic cell colonies. (B) Protein expression pattern is similar to category 3. (C) Protein expression pattern is similar to category 4. (C) Diminished expression of Sall 4 protein in nuclei of embryo fibroblast.

4 CONCLUSIONS

4.1 SUMMARY OF RESULTS

Studies were carried out on the protein expression of two pluripotency genes Oct4 and Sall4. The protein expression employing immunocytochemistry was carried out in different zebrafish embryo developmental stages. Results demonstrated that there were different staining patterns for protein localization in cells. Different developmental stages were then placed in these groupings. The staining patterns were analysed on the basis of protein size, its localization pattern in cytoplasm or nucleus. Based on these analyses a peak expression of both Oct4 and Sall4 was observed in blastomeres isolated from high stage zebrafish embryos. The protein clump size of both Oct4 and Sall4 was maximum (>2μm) at this stage. Likewise the protein clumps were present compactly throughout the nucleus and almost whole area of the nucleus was stained with the protein. It can be assumed that cells are most pluripotent at this stage as compared to other blastula stages i.e. 128-cell stage, oblong stage, dome stage and 50% epiboly. The protein expression patterns at these stages demonstrated either the protein has started expressing or has started to degrade. At 128-cell stage there was cytoplasmic protein localization and protein speck size of both Oct4 and Sall4 was around 0.1 μm which can be maternally expressed protein. Zebrafish embryos undergo mid blastula transition (MBT) at 512-cell stage (Kimmel et al, 1995). Consequently a peak protein expression was observed at high stage which follows 512-cell stage. The later zebrafish embryo stages i.e. oblong stage, dome stage and 50% epiboly showed a reduced protein expression evident from decrease in protein size and sparse localization in nucleus. These findings suggest that Oct4 and Sall4 can be suitable pluripotency markers in zebrafish specie.

The outcomes of the experiments for the expression of pluripotency genes led to the identification of a suitable blastula stage i.e. high stage for the isolation of pluripotent cells. Embryonic cell isolated from high stage were culture in LDF medium. Cell growth was evaluated at two FBS concentrations i.e. 5% and 10%.

Embryonic cell colony diameter and colony no. indicated better growth when 10% FBS is used. Experiments were then carried out on the effect of bFGF on differentiation inhibition in embryonic cell colonies. There was significant difference in neurite forming colonies in bFGF supplemented and non-bFGF supplemented medium. Cell growth was evaluated on the basis of embryonic cell colony diameter and colony no. There was no significant effect of bFGF found on growth of embryonic cell colonies.

The developed pluripotency markers were then analysed on cultured embryonic cells to check their suitability for identification of pluripotent nature of cultured embryonic cell. The immunostained embryonic cell colonies exhibited different protein expression patterns which can be placed in the categories identified for the zebrafish blastomeres. The Oct4 and Sall4 protein expression pattern of cultured embryonic cell colonies identified that these colonies had already start to differentiate. This is in line with our cell culture results which indicated that embryonic cell colonies started exhibiting signs of differentiation i.e. neurite formation, fibroblasts appearance from Day 3 onwards of culturing.

4.2 CONCLUSION

The results from the present study showed that the maximum expression of pluripotency genes Oct4 and Sall4 was found in blastomeres isolated from high stage zebrafish embryos. Oct4 and Sall4 are well studied pluripotency markers in human and mouse, but there is not much reported work on the protein expression of these markers in zebrafish. It is for the first time the expression of these proteins at different developmental stages of zebrafish embryos is reported. The results showed that the blastomeres at high stage have the highest pluripotent nature and zebrafish embryonic cell cultures should be derived from this embryo developmental stage.

Secondly bFGF was found to inhibit neurite formation in our cultured cells, though it has been previously reported that bFGF inhibited melanogenesis and stimulated proliferation in cultured zebrafish embryonic cells (Bradford et al, 1994). Likewise Singh et al (2001) reported suppression of neural markers in

bFGF supplemented zebrafish embryonic cell cultures. The morphological effect of bFGF in inhibiting neurite formation is reported here for the first time.

The applicability of developed pluripotency markers Oct4 and Sall4 was also tested on cultured embryonic cells. Although the staining patterns showed that embryonic cell colonies started to differentiate in our cultures from Day3 onwards, it confirmed the pluripotency markers worked well in identification of the pluripotent nature of embryonic cell colonies. Furthermore the staining patterns of fibroblast which are terminally differentiated cells is very similar to the staining patterns of blastomeres at 50% epiboly stage when cell fate determination has occurred and cells have lost pluripotency. Overall these markers are well suited for pluripotency determination in zebrafish and the findings from the present study will have important application in future zebrafish ES cell culture development.

4.3 FUTURE WORK

More detailed studies should be carried out on the analysis of other pluripotency genes reported in mouse and human species which would help in identifying more suitable culture conditions for zebrafish embryonic stem cells. To further confirm the expression of Oct4 and Sall4 at different zebrafish blastula stages analysed, relative gene expression studies should be carried out employing real time PCR.

In these studies the application of the pluripotency markers was evaluated on cultures in non-bFGF supplemented medium where embryonic cell colonies had already started to differentiate. The applicability of the markers should also be tested on the cultures in bFGF supplemented medium. In the case that colonies are pluripotent, pluripotent cells can be reliably isolated from zebrafish embryo at high stage. More studies should also be carried out on identifying more suitable culture conditions for derivation of embryonic cells from high stage.

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