DNA Methylation Analysis of Sox2 Regulatory Regions SRR1 and SRR2 in Undifferentiated and Differentiated Mouse Embryonic Stem Cells

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Abstract

One hallmark of embryonic stem cells (ES) is their ability to renew themselves indefinitely and still retain the potential to develop into any specialized cell type once triggered by specific exogenous signals. This very versatile nature has made them an attractive model to study developmental events occurring during embryogenesis and also to employ them for regenerative medicine. The idea to exploit their developmental potential for intended therapeutic applications requires a detailed knowledge of the molecular regulation of differentiation. Thus pluripotent embryonic stem cells can be employed to investigate the molecular framework regulating pluripotency. The major aim of this research endeavour is to explore the role of DNA methylation in regulation of endogenous Sox2 transcription factor in context of mouse ES cells following their transition from the pluripotent to a differentiated state. An insight into molecular regulatory mechanisms will shed light on developmental programming and also aid in refining of methodologies for differentiation and nuclear reprogramming increasing their chances of success and efficiency.

Mouse embryonic stem cells were differentiated towards osteogenic and neural cell types through the formation of embryoid bodies (EBs) – cellular aggregates partially recapitulating the early embryonic development. These EBs were then disaggregated and single cells plated in medium containing supplements to promote osteogenic or neural differentiation while control cells were grown in medium lacking those factors. Cells were harvested undifferentiated and at different time points during differentiation. Molecular characterization was carried out by expression profiling of lineage specific genes and proteins using RT-PCR and immunofluorescence respectively. DNA methylation analysis of two regulatory regions of Sox2 i.e. SRR1 and SRR2 was carried out by MS-PCR and bisulphite sequencing.

Embryonic stem cells were observed to be differentiating as evidenced by changes in cellular morphologies and lineage-specific markers expression. Two regulatory regions of Sox2, namely SRR1 and SRR2, were found to be methylated by methylation sensitive PCR at all time-points chosen for analysis in differentiating cells. Three individual CpGs in SRR2 region were then analysed further by bisulphite sequencing which appeared unmethylated in both undifferentiated and differentiated embryonic stem cells. This hints towards the possible role of DNA methylation in regulating the expression of Sox2 in differentiating embryonic stem cells and need further investigation.

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Chapter 1 - Introduction

1. Introduction

Embryonic stem cells are master cells having the potential to develop into any of about 200 different cells types a multicellular creature is composed of and, due to this very versatile nature, are an attractive candidate in regenerative medicine (Prelle et al., 2002). The first successful attempt of in vitro culture of embryonic stem cells with their subsequent maintenance and propagation outside a living system in undifferentiated state has opened a new arena for researchers to explore developmental pathways at one end, and a new hope for millions of those suffering from debilitating disorders at another (Evans and Kaufman, 1981; Martin, 1981).

The reprogramming of somatic genomes to a pluripotent state at will using transcription factors now termed as "core regulatory circuitry" i.e. Oct3/4, Sox2, Myc-4 and Klf-4 indicates the central role of transcription factors in the maintenance of differentiation potential. These transcription factors together with DNA methylation, chromatin modifications and regulatory RNAs result in interactive and extended epigenetic network of regulatory mechanisms (Jaenisch and Young, 2008). Thus pluripotent stem cells can be employed to understand epigenetic changes that accompany cell differentiation and how they contribute to the regulation of differentiation into multiple lineages.

1.1. Stem Cells – Classification and Sources

Stem cells can be broadly classified in to two categories: embryonic or adult (somatic) depending upon their origin of extraction. In mammals, the term "ES cell" refers to stem cells derived from mammalian embryo in general and was specifically coined to distinguish these

cells from "EC cells" – teratocarcinoma derived pluripotent embryonal carcinoma cells which are similar to them in morphology, growth behaviour and marker expression (Martin, 1981). ES cells are also able to produce teratocarcinoma but they tend to consists of well differentiated mesodermal, ectodermal and endodermal tissues and cell types in contrast to those derived from EC cells that are dominated by undifferentiated cells (Evans and Kaufman, 1983).

Embryonic stem cells are derived from the inner cell mass (ICM) of the blastocyst stage of embryo. The surrounding tissues of the ICM form the trophoblast which ultimately froms into placenta and other supportive tissues required for fetal development (Wobus and Boheler, 2005). Pluripotent stem cells give rise to somatic stem cells of limited differentiation potential and hence referred to as multipotent stem cells which can develop into specified cell types of a particular lineage such as haematopoietic stem cells, neural stem cells etc. as shown in Figure 1:

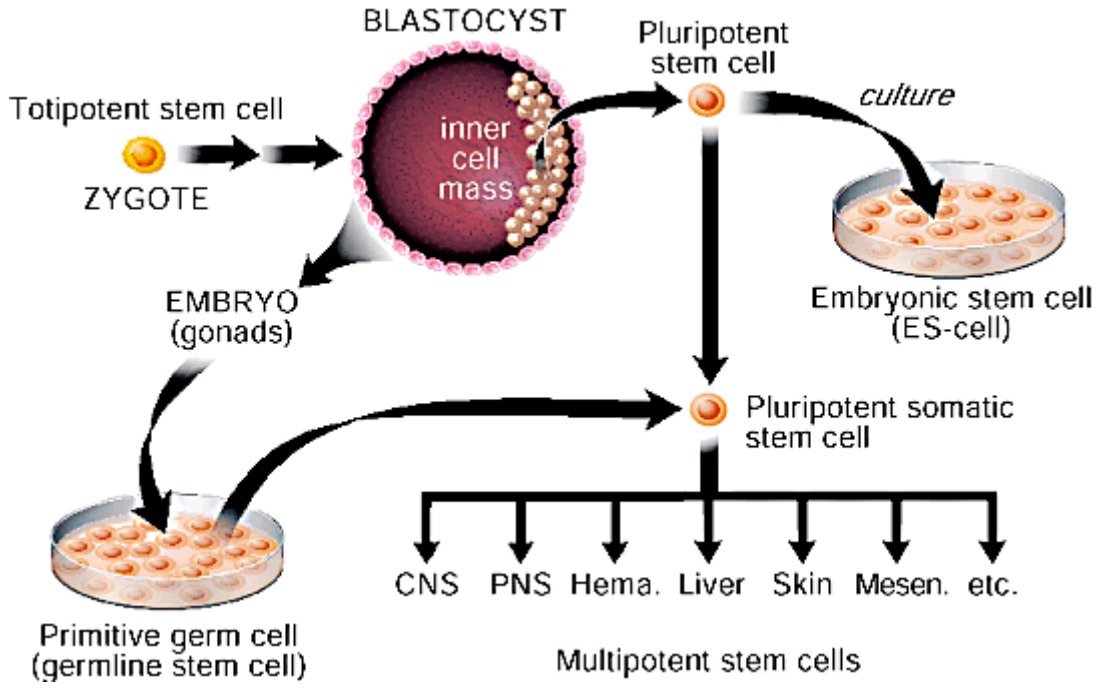


Figure 1: Classification of stem cells based on their differentiation potential (Anderson *et al.*, 2001)

Multipotent stem cells are localized to tissues or organs and are generally regarded as adult or somatic stem cells to differentiate them from their embryonic counterparts. These cell populations mainly act as an active or passive reservoir to renew depleting/dying/damaged cells based on their localization and therefore serve as a continuous source of cell renewal inside a living system (Anderson et al., 2001). It was believed that these cells could have limited differentiation potential restricted to one lineage but recent evidence indicates that under appropriate conditions they can differentiate across different lineages, a phenomenon known as transdifferenatiation (Blau et al., 2001).

1.2. Mouse Embryonic Stem Cells - Derivation and Maintenance

Mouse is an established animal model for experimental studies in mammals because of its genetic and physiological similarity with human beings (Fields and Johnston, 2005). Mouse embryonic stem cells are derived from inner cell mass of blastocyst stage embryo. Tissue culture medium enriched with 10-20% fetal calf serum and 2-mercaptoethanol is routinely used for their propagation either with or without feeders (Smith, 2001; Robertson, 1987). Mouse ES cells propagation and maintenance in an undifferentiated and pluripotent state is also dependent on the presence of LIF, Leukaemia inhibitory factor, so called because it was originally isolated as a haemopoietic regulator (Williams et al., 1988; Smith et al., 1988). LIF is a cytokine of the IL-6 family and acts through the gp130 receptor resulting in the activation of a series of signalling pathways involving STAT3, SHP-2, Hck, ERK1 and ERK2 (Burdon et al., 1999a, b). The exact role of every single factor and signalling pathway in keeping the self-renewal ability is not known yet (Smith, 2001). A pictorial representation of different stages of mouse embryogenesis and stem cells at different time points is shown in Figure 2:

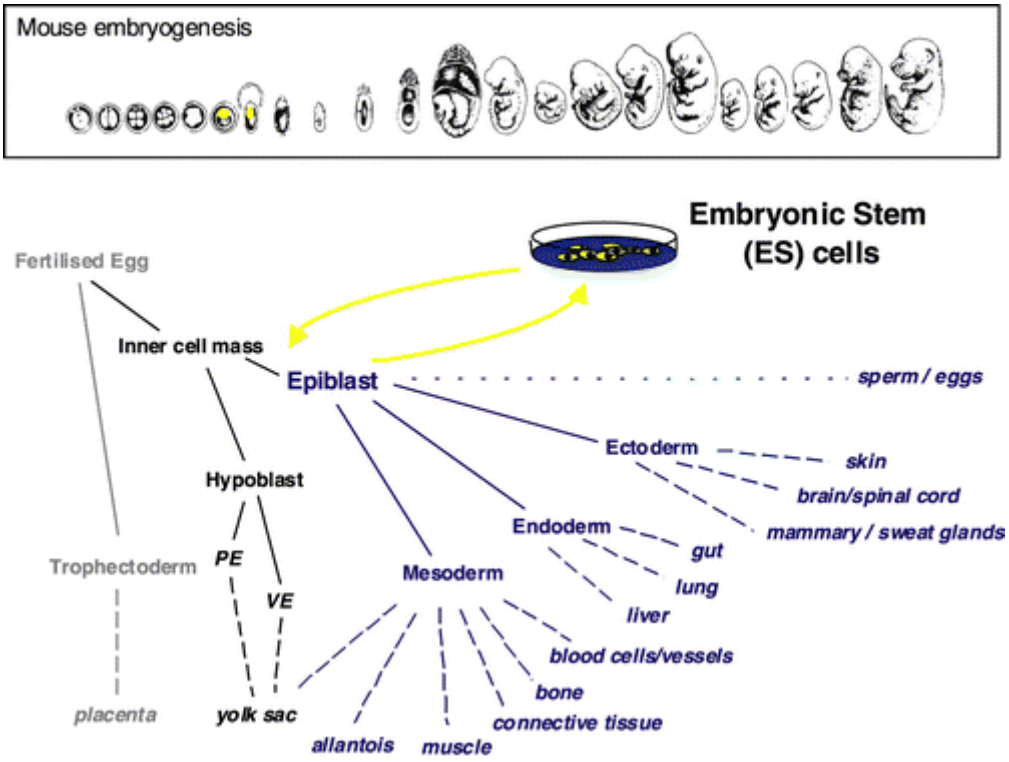


Figure 2: Developmental pathway in mouse (upper panel) and ES cells at different stages (lower panel) in this context (Smith, 2001)

One of the rationales for growing embryonic stem cells in vitro is to create an environment similar to that found inside the embryo to understand complex developmental events. But development is largely a context-dependent phenomenon involving cell-cell interactions at tissue and organ level besides various environmental stimuli. So functional assessment criteria are needed to interpret and validate the information obtained from in vitro studies to complement that with real physiological systems. The lowest level of this functional assessment regarding the validation of pluripotent state of embryonic stem cells is firstly achieved through in vitro differentiation of cultured cells. This is followed by teratoma formation after injection into host and then contribution to germ line by injected cells and chimera formation. The extreme level of control is exercised by injection of laboratory grown cells into 4n host blastocyst to get an animal composed only of injected donor cells (Jaenisch and Young, 2008; Eggan et al., 2001; Nagy et al., 1990). The ability to produce chimeras or

to validate germ line contribution for humans is not possible due to ethical and technical reasons hence primate ES cell lines other than human are used as an accurate in vitro model to understand differentiation (Thomson et al., 1995; Thomson et al., 1998). Also the process of gastrulation and germ layer formation are difficult to characterize in vivo due to the small size of the embryo and rapid start of development after implantation in the uterus. Embryonic stem (ES) cells are derived from the inner cell mass of early blastocysts or from the epiblast of late blastocysts (human ES cells and mouse EpiES), and have been shown to be able to self-renew or differentiate in most tissues of an adult organism under specific culture conditions in vitro, therefore providing an attractive model to understand pluripotency and its regulation at a molecular level (Smith, 1992; Pesce et al., 1999).

1.3. "Pluripotency" or "stemness" of mouse ES cells at molecular level

Pluripotency is a cellular state of embryonic stem cells characterized by their potential to differentiate into cell types originating from all three germ layers of a developing embryo i.e. ectoderm, mesoderm and endoderm (Niwa, 2001). A complex and dynamic interplay mediumted by a diverse range of networks has been shown to contribute towards pluripotency rather than a single universal mechanism. It comprises of transcription factors, chromatin modifiers, regulatory RNAs and signalling pathways which are generally described together as epigenetic regulators of pluripotency (Jaenisch and Young, 2008).

Conard Waddington (1905-1975) is credited for introducing the term epigenetic and he defined it as, "the branch of biology which studies the casual interactions between genes and their products, which bring the phenotype into being" (Waddington, 1942; Jablonka and Lamb, 2002). An updated and currently prevailing definition of epigenetic is, "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by

changes in DNA sequence" (Bird, 2007). Hence epigenetic in its true essence is a sort of bridge between genotype and phenotype to explain any observed disparity in terms of phenotypic outcome at a particular locus or chromosome but without associated DNA sequence changes (Goldberg et al., 2007).

Epigenetic influences on cell fate decisions are inherited in a non-Mendalian way in contrast to genetic changes and are usually triggered by a signal event (Rassoulzadegan et al., 2006). There are many allowed combinations of epigenetic marks to be taken by a cell during differentiation and Figure 3 shows a visual representation of epigenetic landscape based on Waddington's idea.

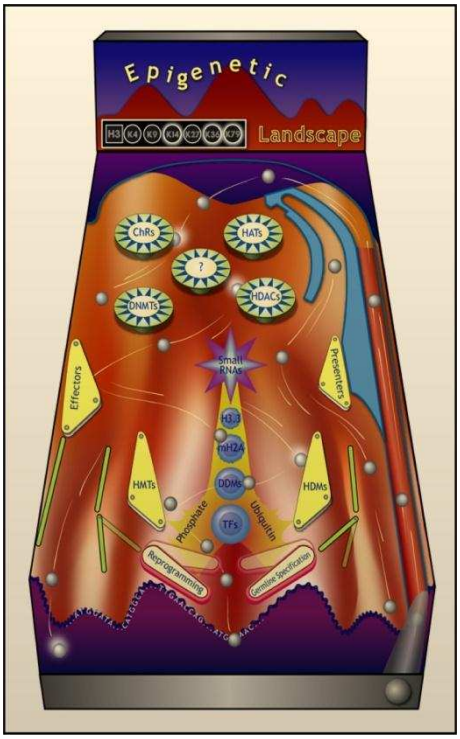


Figure 3: A current view of the epigenetic molecular machinery. Known factors that regulate epigenetic phenomena are shown directing the complex movement of pinballs (cells) across the elegant landscape proposed by Waddington. No specific order of molecular events is implied; as such a sequence remains unknown. Effector proteins recognize specific histone modifications, while presenters are proposed to impart substrate specificity for histone-modifying enzymes (Ruthenburg *et al.*, 2007). H3.3 and macroH2A are shown only as representative histone variants involved in transcriptional activation or repression, respectively. For simplicity, other histone (and non-histone) proteins are not shown. (Key: ChR, chromatin remodelers; DNMTs, DNA methyltransferases; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; HDMs, histone demethylases; DDMs, DNA demethylases [unidentified in mammals to date]; and TFs, transcription factors [reflecting the genetic component of the epigenetic process]). Text and figure are from (Goldberg *et al.*, 2007)

After the cell has decided to take on a specific differentiation pathway in response to an external stimulus, self-perpetuating epigenetic memory allow cells to maintain their identity through the involvement of polycomb proteins even in the absence of inducing signal (Ringrose and Paro, 2004). This is also true for those environments that are rich in signals favouring induction of other cell types, hence important in maintaining the identity of stem cells over time (Martin and Zhang, 2007). This epigenetic memory is a specific cell state comprised of selective expression of some transcription factors acting in conjunction with chromatin modifications – covalent modifications of histone tails, DNA methylation at CpG dinucleotides, and localization of chromatin to specialized nuclear domains (Li, 2002).

1.3.1 Transcription Factors

The process by which genetic information encoded in double helix of DNA is expressed through the mediumtion of mRNA is called transcription and is facilitated by transcription factors to a large extent (Watson et al., 2008). Transcription factors can be grouped into two categories based on their expression: basal transcription factors or general transcription factors which are expressed ubiquitously and, regulatory transcription factors expressed in tissue-specific manner (Lee and Young, 2000). Those associated with expression of tissue-specific genes have been found to act via different interacting positive and negative feedback loops on their target to maintain irreversible cell differentiation. They act on multiple differentiation pathways in temporal order to control and co-ordinate their output through self-maintained nuclear networks (Schulaz and Hoffmann, 2007).

The transcription factor network involved in the maintenance of pluripotent state in embryonic stem cells has been studied in detail (Boyer et al., 2005; Loh et al., 2006). This network is suggested to be ranked at higher position in a hierarchy of pluripotency controlling

elements (Silva and Smith, 2008) as only a trinity of factors, Oct4, Sox2 and Nanog referred to as "core regulatory circuitry", are required to maintain that state (Chambers and Smith, 2004; Niwa, 2007).

Oct4 is a member of the POU-family of transcription factors, it is encoded by Pou5f1 and is shown to be expressed in totipotent and pluripotent cells in the mouse life cycle (Pesce et al., 1998a). Targeted gene deletion experiments in mouse embryos have resulted in failure of fetal development indicating its importance in pluripotency as cells differentiated to trophectoderm lineage (Nichols et al., 1998). Oct4 can heterodimerize with Sox2 – an HMG box transcription factor which in turn regulates the Oct4 expression level (Masui et al., 2007). Nanog has been shown to stabilize the pluripotent state rather than being essential for its maintenance (Chambers et al., 2007).

These three factors bind together at their own promoters and form an interconnected auto-regulatory loop besides co-occupying their target genes. They collectively target two sets of genes, one that is actively expressed and another that is silent in embryonic cells but remain poised for expression during differentiation (Boyer et al., 2005; Jaenisch and Young, 2008). The majority of genes occupied by these master regulators of pluripotency are those of transcription factors, signal transduction components and chromatin modifying enzymes that promote ES cell self-renewal. But they also co-occupy and silence genes whose products are required in lineage commitment and cellular differentiation hence maintaining stemness of ES cells (Boyer et al., 2006; Loh et al., 2006).

Most of the developmentally silent promoters are also occupied by Polycomb group (PcG) proteins that maintain cell state through gene silencing and hence are epigenetic regulators

(Bernstein et al., 2006; Lee et al., 2006). These proteins form multiple polycomb repressive complexes (PRCs) which mediumte gene silencing at the transcription level (Schuettengruber et al., 2007). The presence of stalled RNA polymerase at promoters of genes targeted by PcG and core transcription regulators involved in developmental regulations suggests that they are prone to transcription once differentiation is triggered (Mikkelson et al., 2007; Bernstein et al., 2006). The mechanisms triggering selective activation of genes encoding specific developmental regulators have not been revealed yet, but most likely involve signaling pathways leading to establishment of a specific signature event such as nucleosome modifications e.g. demethylation of core histone 3 at lysine 27 (Lan et al., 2007).

1.3.2 Chromatin Modifications

The majority of DNA in mammalian cells is found in a condensed state called chromatin which is composed of a basic repeating unit called the nucleosome. In all eukaryotic cells each individual nucleosome consists of an octamer of four core histone proteins (H2A, H2B, H3 and H4) around which 147 base pairs of DNA are wrapped. Nucleosomes are linked together by linker DNA whose length is species dependent but typically fall in the range of 20-60 bps and can be bound by another histone protein H1 usually referred to as linker histone (Kornberg and Lorch, 1999).

Histones are positively charged proteins rich in basic amino acids i.e. lysine and arginine, and heterodimerization of specific histone pairs is mediated by a conserved histone fold domain. Histone proteins pairs get an ordered structure only after binding to DNA in a sequential manner; first a H3.H4 tetramer binds DNA which is successively joined in by two dimers of H2A.H2B to form nucleosome. These core histones have amino-terminal extensions called “histone tails” which are not required for the formation of nucleosome (Luger et al., 1997),

but are indispensable for nucleosome remodelling and accessibility of DNA to replication and transcription machinery. These tails are targets for different type of dynamic covalent modifications which give the nucleosome an individual function and are mostly modified by phosphorylation, acetylation and methylation on serine, lysine and argininie residues (Strahl and Allis, 2000). These modifications appear in different combinations on a variety of target amino acids in histone tails depending on the set of conditions and they confer cells an enormous functional diversity to keep up with the changing cellular contexts (Kouzarides, 2007).

The observation that some combinatorial patterns of histone tail modifications have been shown to display identical phenotype (e.g. acetylation at certain specific amino acids is correlated with transcriptional upregualtion in most instances) has led to the emergence of the "Histone code" concept. This code symbolizes the extension of genetic code at post genetic level and the enzymes carrying out these reactions are highly specific for their target amino acids with respect to changes and positions thus generating interdependent series of modifications (Strahl and Allis, 2000). This concept has been further extended to "nucleosome code" whereby concentration and combination of differentially modified individual nucleosomes in higher order chromatin structure dictates multiple readouts of genetic information e.g. gene activation v/s silencing or cell proliferation v/s differentiation (Jenuwin and Allis, 2001). But discrepancies have been reported between combination of signal events and generated output limiting the use of the term code in a universal sense (Liu et al., 2005). A brief account of common histone modifications is detailed below.

1.3.2.1 <u>Acetylation</u> at lysine (represented by K) residues has been the most studied modifications of histone tails so far. One possible reason might be the

concomitant discovery of enzymes maintaining a balance between acetylated and non-acetylated state of target residues namely histone acetyl transfersases (HATs) and histone deacetylases (HDACs) and also the observation that it is almost invariably associated with transcription activation (Gurnstein, 1997; Struhl, 1998). HAT is an enzyme that catalyses the acetylation of specific lysine residues in core histones resulting in changes in chromatin structure compactness and hence, effecting gene expression (Lee and Workman, 2007). Acetylation of lysine residues neutralizes the positive charge of histone tails leading to weak histone-DNA, nucleosome-nucleosome, histone-regulatory protein interactions making chromatin environment more permissive for transcription (Masumi, 2011; Roth et al., 2001). Most common sites observed being acetylated in histones are K9, K14, K18 and K23 in H3 and K5, K8, K12 and K16 in H4 in most species (Thorne et al., 1990; Strahl and Allis, 2000).

p300/CBP (CBP is phosphorylated form of cAMP-response-element-binding protein) is example of the two most studied HATs which were identified as separate protein factors initially but later grouped together due to structure and functional relatedness (Roth et al., 2001). Several distinct domains are present in p300/CBP including a bromodomain (a frequent feature of mammalian HATs), three histidine-cysteine rich domains (mediates protein-protein interactions) and two more domains that interact with different transcription factors e.g. c-Jun, nuclear hormone receptors, TFIID (a general transcription factor). p300/CBP are recruited to their target by interactions with DNA-bound transcription factors instead of directly binding to the DNA and regulate many different transcription and signalling pathways (Chan and Thangue, 2001; Roth et al., 2001).

1.3.2.2 Phosphorylation occurs at serine (represented by S) and sometimes on threonine (marked as T) residues and specifically this modification on H1 and H3 histone tails is associated with chromosome condensation during mitosis (Bradbury, 1992; Koshlan and Strunikov, 1996). H3S10 phosphorylation is specifically associated with upregualtion of "immediumte early" genes such as c-fos, c-jun and c-myc (Thomson et al., 1999; Chadee et al., 1999). This modification has been reported to have most significant influence on signalling pathways. The phosphorylation of cytoplasmic proteins initiates a cascade of signalling networks which in turn affects gene expression e.g. mitogen activated protein (MAP) kinase pathway which is linked with H3 phosphorylation (Thomson et al., 1999).

1.3.2.3 Methylation has been shown to occur on arginine and lysine residues in histone N-terminal chains and output effects can be either activating or repressive on transcription based on location of residues methylated and groups of methyl added to target residue i.e. mono, di or tri (Bannister and Kouzardies, 2005). This metylation is catalyzed by three distinct families of proteins: protein arginine methyl transferase (PRMT1) family, SET domain containing proteins and DOT1/DOT1L histone methyl transferase family (Martin and Zhang, 2005). Methylation of H3K4, H3K36 and H3K79 is associated with active genes while H3K9, H3K27 and H4K20 are found to be related with transcription repression (Strahl et al., 1999). An interesting feature is differential marking of methylation pattern i.e. H3K4me3 associated with transcription start sites, H3K36me3 with gene sequences, H3K9me3 abundant on silent genes and H3K36me3 on exons but at lower levels on alternatively spliced exons (Kolasinska-Zwierz et al.,

2009). The consequences of arginine methylations are not well understood so far and PRMTs – protein arginine methyl transferases have been found to be recruited to promoter by transcription factors (Lee et al., 2005a, b) but no protein has been identified to reverse arginine methylation (Kouzarides, 2007).

1.3.2.4 Ubiquitylation has been shown to occur on H2AK119 and H2BK20. This modification is mediumted by the Bmi/Ring1A protein found in polycomb complexes and is associated with transcriptional repression (Wang et al., 2006). SUMO (small ubiquitin-related modifier) is a small protein having three dimensional structure similar to that of ubiquitin but distinct cellular functions (Hay, 2001). Sumolyation have been shown to modulate protein-protein interactions, nuclear localization of subcellular components, chromatin organization, protein-DNA interactions, enzyme activity, and as antagonist of ubiquitylation which marks proteins for degradation (Melchior, 2000; Verger et al., 2003; Johnson, 2004). It has also been shown that histone H4 modification by sumolyation lead to transcription repression through recruitment of histone deacetylases (HDACs) and heterochromatin protein 1 (HP1) both in vivo and in vitro (Shiio and Eisenman, 2003). HDACs catalyse the acetyl group removal from the histone tail residues, acetylation is a modification almost invariably associated with transcription activation as described above in section 1.3.2.1. HP1 is a known structural protein of heterochromatin that binds to methylated lysine (K) of H3 and leads to transcriptional repression (Nielsen et al., 2001). Histone sumolyation is also reported to act as negative regulator of transcription in Saccharomyces cerevisiae (Nathan et al., 2006).

There are two proposed mechanisms by which histone modifications function: by disruption of nucleosomes-DNA contact in chromatin and/or by recruiting non histone proteins having enzymatic activities which depending upon the type of modifications that recruited them will facilitate the unfolding or the compaction of nucleosomes thus affecting downstream cellular processes (Kouzarides, 2007). The transcriptionally active chromatin is referred to as euchromatin and is characterized by high level of acetylation and trimetylation mainly at H3K4, H3K36 and H3K79. By contrast, the term heterochromatin is used to describe the chromatin with little or no transcription and associated with low levels of acetylation, increased methylation at residues associated with silent loci and phosphorylation (Strahl and Allis, 2000). These two regions are demarcated by boundary elements in mammals because each is associated with distinct modifications which are maintained to preserve identities of these domains.

Boundary elements are cis-acting sequences in genomes of higher eukaryotes that define the distinct functional domains of genome by acting as a barrier to prevent spread of inactive heterochromatin into euchromatin and also restrict the action of regulatory elements to the appropriate target region (Mishra and Karch, 1999). Similar kind of elements are also proposed to be present in CpG islands that protect them from de novo DNA methylation and induce local demethylation during development. They are thought to contain binding sites for other proteins e.g. Sp1 and, together they form a physical barrier preventing the binding of other regulatory elements and methylating enzymes to the target sequence (Brandeis et al., 1994; Macleod et al., 1994). Sp1 is a ubiquitous transcription factor that regulates a diverse set of genes involved in cell differentiation, signal transduction and apoptosis along with housekeeping genes. Binding sites for Sp1 and related transcription factors (GC/GT box is the consensus binding site) are frequent in regulatory sequences such as promoters, enhancers

and CpG islands of genes (Bouwman and Philipsen, 2002). The boundary elements also recruit enzymes to modify the associated chromatin e.g. PC2 is recruited by H3K27me to maintain inactive X chromosome (Plath et al., 2003; Umlauf et al., 2004). Furthermore, histone modifications are not static cis acting phenomena rather dynamic and co-operative process where many cross talks between histone tail residues facilitate binding of a target protein but can at same time disrupt the binding of another repressor /activator on adjacent residue due to steric hindrances (Kouzarides, 2007).

The pluripotent state of embryonic stem cells is maintained mainly by polycomb proteins and trithorax proteins which are involved in mitotic inheritance of lineage specific genes and, have been shown to catalyse H3K27 and H3K4 methylation respectively (Ringrose and Paro, 2004). H3K4 trimethylation has been reported as a signature event resulting in recruiting histone acetylases signalling up regulation of transcription while H3K27 has been found to down regulate transcription by compacting chromatin (Bernstein et al., 2005). These two contrasting signatures have been shown to coexist in highly conserved non coding elements (HCNEs) in the mammalian genome and so named “bivalent domains”. These regions encode transcription factors required for embryonic development and their existence as bivalent domains keeps expression of these TFs at low level yet prone to transcription if ES cells differentiate. Once ES cells have taken any differentiation pathway, only one of the mark is perpetuated either repressive H3K27 or activating H3K4 (Bernstein et al., 2006). This finding has opened up a possibility for manipulating the pluripotent state of embryonic stem cells by altering the regulatory balance of these modifications. However, the overall structure of chromatin in ES cells and how it is linked to pluripotency maintenance have not been detailed yet (Szutorisz and Dollin, 2005).

1.3.3 DNA Methylation and CpG Islands

DNA methylation is an epigenetic modification associated with repression of tissue specific genes, genomic imprinting, X chromosome inactivation and transposon silencing, and in mammals occurs almost invariably at position 5 of the Cytosine ring found in CG dinucleotides (Bester and Bourc'his, 2004; Bortvin et al., 2003; Li, 2002). CpG islands refers to those GC rich regions (65%) of about 1kb length that are methylation free and found to be associated with promoters of 50% of all mammalian genes (Antequera and Burd., 1993), making them a reliable feature for promoter prediction in mammalian genome (Hannenhalli and Levy, 2001; Ioshikhes and Zhang, 2000). CpG islands are enriched in transcription factor binding sites, have an open chromatin conformation and are different from the rest of the genome which is GC poor (40% on average) and heavily methylated at CpG dinucleotide (Bird, 1986; Delgado et al., 1998).

These islands were originally referred to as "HTF islands" – "HpaII tiny fragment fraction" owing to their sensitivity to the HpaII (CCpGG) restriction enzyme. HpaII cuts at methylated CpG and sensitivity to this enzyme was found to be associated with promoters of housekeeping genes and some of tissue specific genes in digested fragments (Bird et al., 1985). The distribution of CG dinucleotide in terms of frequency ratio is 1 in CpG islands in contrast to 0.2 observed in the rest of the genomic DNA. This disparity points towards a direct consequence of methylation in CpG islands. Unmodified cytosine is prone to deamination which converts it to uracil and is removed by uracil-glycosylation by the DNA repair machinery (Lindahl, 1982). By contrast, deamination of methylated cytosines converts them to thymines and results in C to T transition mutations that remain fixed as TpG (or CpA on the complimentary strand) by the DNA repair system thus accounting for the high

frequency of mutation rates observed at CpGs (Bird, 1980). That explains why CpG islands have displayed a more stable existence over the course of evolution as the U:G mismatch is removed far more efficiently and generation of new CpG sites by point mutations counter balance to maintain 20% of the expected frequency (Sved and Bird, 1990).

Methylation of CpG islands is found to be associated with transcription repression highlighting the importance of methylation and its maintenance at those sites (Keshet et al., 1985). Enzymes mediumting methylation of CpG islands are generally classified in two categories: de novo methyltransferase (DNMT3a and DNMT3b) which adds a methyl moiety at a cytosine residue and maintenance methyltransfersase (DNMT1) which copies the existing methylation pattern onto newly replicated DNA molecule hence keeping the methylated status over successive cell divisions (Klose and Bird, 2006).

De novo methylation activity has been shown to occur mainly in ES cells, embryonic carcinoma cells, early post implantation embryo and developing germ cells and is absent in differentiated somatic cells (Chen et al., 2003). How de novo methyltransferases are recruited to their target during germ line development to establish methylation patterns has not been characterized in detail due to unavailability of biological material at that stage (Reik et al., 2001). Cell culture systems therefore have been widely exploited to understand this mechanism and three possible explanations have been put forward: the DNMT3 enzymes might recognize targets via specific domains in their structure, or they may be recruited to their targets by protein-protein interactions, or through RNA interference mediumted mechanisms (Klose and Bird, 2006). Mouse embryonic stem cells lacking DNA methylation can survive and proliferate in an undifferentiated state but undergo apoptotic cell death upon differentiation. Therefore it remains to be determined whether this methylation is involved in

gene expression or maintenance of pluripotent state highlighting the importance of this event particularly if ES cells have to be employed in regenerative medicine (Fouse et al., 2008).

Two mechanisms have been proposed for gene repression by methylation: either the modification of target sequence abolishes the target site of DNA binding factors (Watt and Molloy, 1988); or methylated sites recruit protein complexes that have transcription inhibitory activity (Handrich and Bird, 1998; Boyes and Bird, 1991). Methyl-CpG-binding proteins (MBPs) are associated with transcriptional repressor molecules that repress transcription with concomitant modification of surrounding chromatin linking DNA metylation and chromatin modification (Klose and Bird, 2006). Histone metylation at H3K4 has been implicated in promoter protection from de novo methylation in somatic cells (Weber et al., 2007) and maternal imprinting control regions (ICRs) are rich in H3K4me2 which become methylated during oogenesis (Delaval et al., 2007). Figure 4 illustrates the possible gene repression mechanism by DNA methylation and how DNA methylation pattern is faithfully copied during replication.

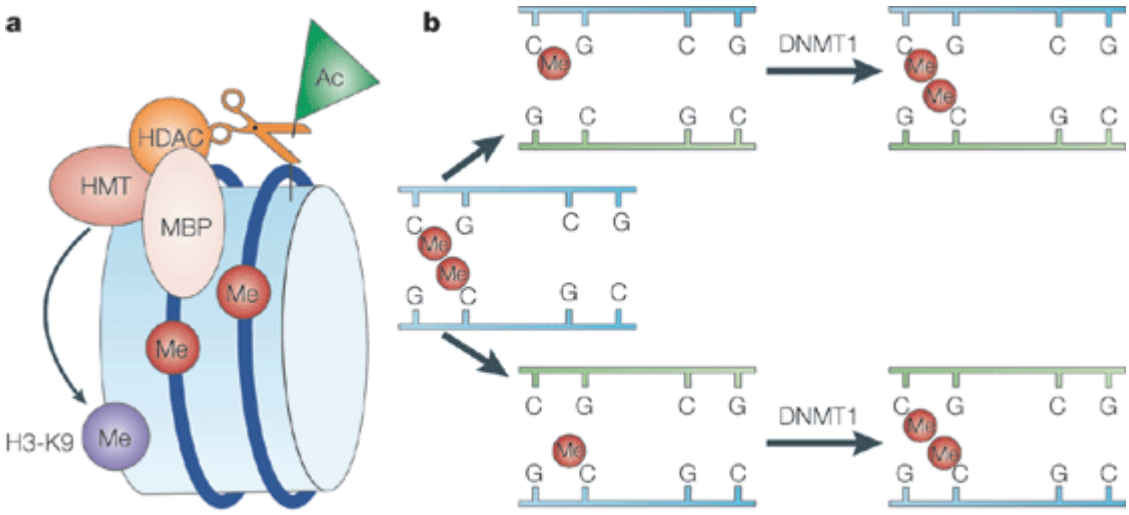


Figure 4: a) DNA methylation of promoters inhibits transcription by several molecular strategies. More commonly, methylated CpGs are recognized by methyl-binding domain proteins (MBP) that can recruit histone deacetylase (HDAC) and histone methyl transferase (HMT) and in this way bring about histone deacetylation and H3K9 methylation that alters the local chromatin environment.
b) During replication, the newly made DNA strand (green) is generated using unmodified cytosine. DNA methyltransferase (cytosine 5) 1 (DNMT1) then progressively modifies only those sites that already have a methyl moiety on the parental DNA strand. In this way, the pattern of methylation is faithfully copied to the daughter cells, where it then contributes to reconstructing the chromatin structure and factor composition at the promoter (Text and Figure from Land-Diner and Cedar, 2005). [Ac, acetylation; Me, methylation.]

1.3.4 Regulatory RNAs

RNA interference is a vast and newly discovered regulatory network of gene expression in eukaryotes sharing functional similarities with prokaryotes (Cerutti and Casas-Mollano, 2006). This is a method by which small non-protein coding RNA molecules of about 21-22 nucleotides interact with mRNA and modulate the expression repertoire of eukaryotic cells (He and Hannon, 2004; Chapman and Carrington, 2007). These non-coding RNAs have been shown to modulate various epigenetic regulatory mechanisms such as chromatin modifications, RNAi-directed DNA methylation leading to silencing of genes, imprinting mainly dosage compensation, cellular decision making and differentiation (Bernstain and Allis, 2005). RNA interference can be classified into three branches based on type of regulatory RNA pathway involved (Matranga and Zamore, 2007):

a) Small interfering RNA (siRNA) pathway providing defense against viruses and transposable elements.

b) Micro RNA (miRNA) pathway which regulates gene expression.

c) Piwi-interacting RNA (piRNA) involved in retrotransposon and germ line gene silencing.

miRNA and siRNA mediumted pathways have some underlying differences. For instance miRNAs are endogenous non-protein coding RNA encoded by genes present in genome while siRNAs are degradation product of exogenous (e.g. virus) double stranded RNAs or transcribed from transposable elements integrated in genome or from inverted repeats or from overlapping and oppositely transcribed gene pairs. siRNAs show absolute complementarity to their target sequence in contrast to miRNAs where complementarity to target is partial particularly in animals (Shabalina and Koonin, 2008). And among these complementary sequence 2-8 residues of miRNA are most conserved among homologous miRNAs (Lim et al 2003).

The mechanism by which transcription and/or post transcriptional regulation of gene expression can be mediumted by miRNA or siRNA depends on the level of complementarity: miRNA or siRNA with high level of complementarity can recruit RNA-induced silencing complex (RISC) and thus direct mRNA degradation; partial complimentary will lead instead to translational repression (Hutvagner and Zamore, 2002; Zeng et al., 2002, 2003; Doench et al., 2003). The choice of the post transcriptional mechanism is therefore independent of the identity of the RNA; rather the sequence similarity to target is the major determinant (Bartel, 2004). After cleavage of mRNA, the miRNA or siRNA remains intact and can further undergo similar cycles of target recognition and destruction (Tang et al., 2003). Figure 5 represents the mode of action of different type of regulatory microRNAs either at

transcription or post transcription level. Endogenous miR-145 has been shown to directly target 3'-untranslated regions of SOX2, OCT4 and KLF4 in embryonic stem cells and switch the cell fate from undifferentiated state to a differentiated state i.e. increase expression of miR-145 represses the expression of these core pluripotency associated genes and induce differentiation (Xu et al., 2009). Another example whereby gene expression is controlled by regulatory RNAs in stem cell is miR-124 which directly target the Sox9 gene, whose down regulation is the key for neural differentiation (Cheng et al., 2009).

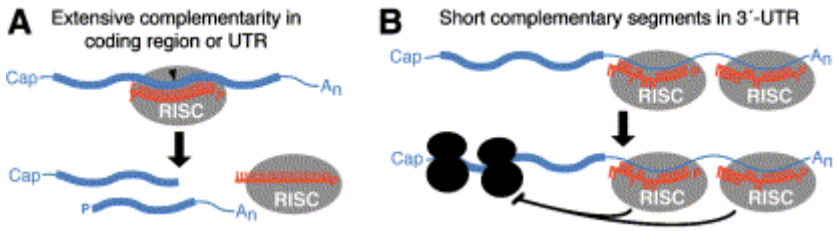


Figure 5: The Actions of Small Silencing RNAs(A) Messenger RNA cleavage specified by a miRNA or siRNA. Black arrowhead indicates site of cleavage.(B) Translational repression specified by miRNAs or siRNAs (Bartel, 2004).

Eukaryotes also express another class of non-protein coding RNAs greater than 200 nucleotides in size known as large non-coding RNAs (lncRNAs) (Kapranov et al., 2007). These lncRNAs can be cytosolic, nuclear, spliced, polyadenylated and originate from either strand within a protein coding locus (Ponting et al., 2009). These lncRNAs have been reported to be playing diverse functional and regulatory roles (summarized in Figure 6) such as chromatin remodeling, telomere biology, subcellular structural organization, transcriptional and post-transcriptional gene regulation (Mercer et al., 2009). Given their diverse roles and cell and tissue specific expression, they are considered to be having crucial roles in development and cellular differentiation and their aberrant expression can also lead to different diseases including cancer (Caley et al., 2010). The most notable example of gene regulation by lncRNA is X chromosome inactivation (XCI) in female cells by Xist lncRNA

that coats one of the X chromosome making it transcriptionally inactive in a phenomenon called dosage compensation (Lee, 2010). Evx1as and Hoxb5/6as ncRNAs have been shown to have a role in epigenetic regulation of homoeotic loci during differentiation of ES cells where both found to be associated with trimethylated H3K4 histones and histone methyltransferase MLL1 (Dinger et al., 2008).

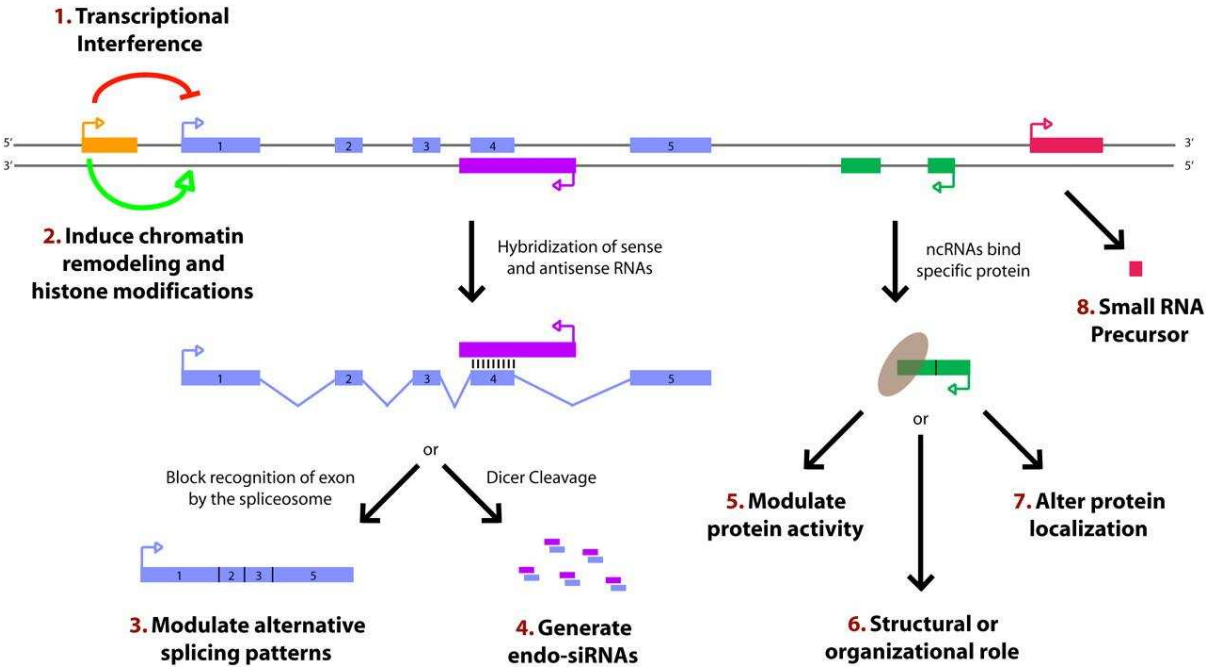


Figure 6: Paradigms for how long ncRNAs function. Recent studies have identified a variety of regulatory paradigms for how long ncRNAs function, many of which are highlighted here. Transcription from an upstream noncoding promoter (orange) can negatively (1) or positively (2) affect expression of the downstream gene (blue) by inhibiting RNA polymerase II recruitment or inducing chromatin remodeling, respectively. (3) An antisense transcript (purple) is able to hybridize to the overlapping sense transcript (blue) and block recognition of the splice sites by the spliceosome, thus resulting in an alternatively spliced transcript. (4) Alternatively, hybridization of the sense and antisense transcripts can allow Dicer to generate endogenous siRNAs. By binding to specific protein partners, a noncoding transcript (green) can modulate the activity of the protein (5), serve as a structural component that allows a larger RNA–protein complex to form (6), or alter where the protein localizes in the cell (7). (8) Long ncRNAs (pink) can be processed to yield small RNAs, such as miRNAs, piRNAs, and other less well-characterized classes of small transcripts. (Text and figure from Wilusz *et al.*, 2009)

1.4 Development and Cellular Differentiation

Development is a sequential process resulting in cellular specification through the process of cellular differentiation i.e. cells become specific in their function and localized in the developing animal body (Davidson et al., 2002). The key of this process is differential control of gene expression mediated mainly by transcription factors and their interactions with cis-regulatory DNA sequence elements e.g. enhancers, silencers, insulators and locus control regions that can be found either upstream, downstream or in introns of a gene (Istrail and Davidson, 2004; Maston et al., 2006). Cis-acting elements are DNA sequences that lie in physical vicinity of the target gene being regulated mostly found in promoters, upstream or downstream regions while trans-acting elements can diffuse to their target and may be a product of a gene locating several base pairs away from the target site (Albert et al., 2002). It is the combinatorial action of these cis-regulatory DNA elements with transcription factors that produces a specific gene expression pattern and/or multiple patterns in spatial and temporal manner corresponding to specific cell type giving its identity (Levine and Davidson, 2005). These complex interactions can be regarded as gene regulatory networks (GRNs) consisting of hierarchy of interactions among transcription factors, cis acting regulatory sequences and signalling molecules (Davidson et al., 2003). A regulatory gene with its multiple control elements is the central hub of these networks that receive inputs and generate output signals in the form of transcription factors and signalling molecules received from and, directed to these interacting regulatory networks as shown below in Figure 7 (Leon and Davidson, 2007).

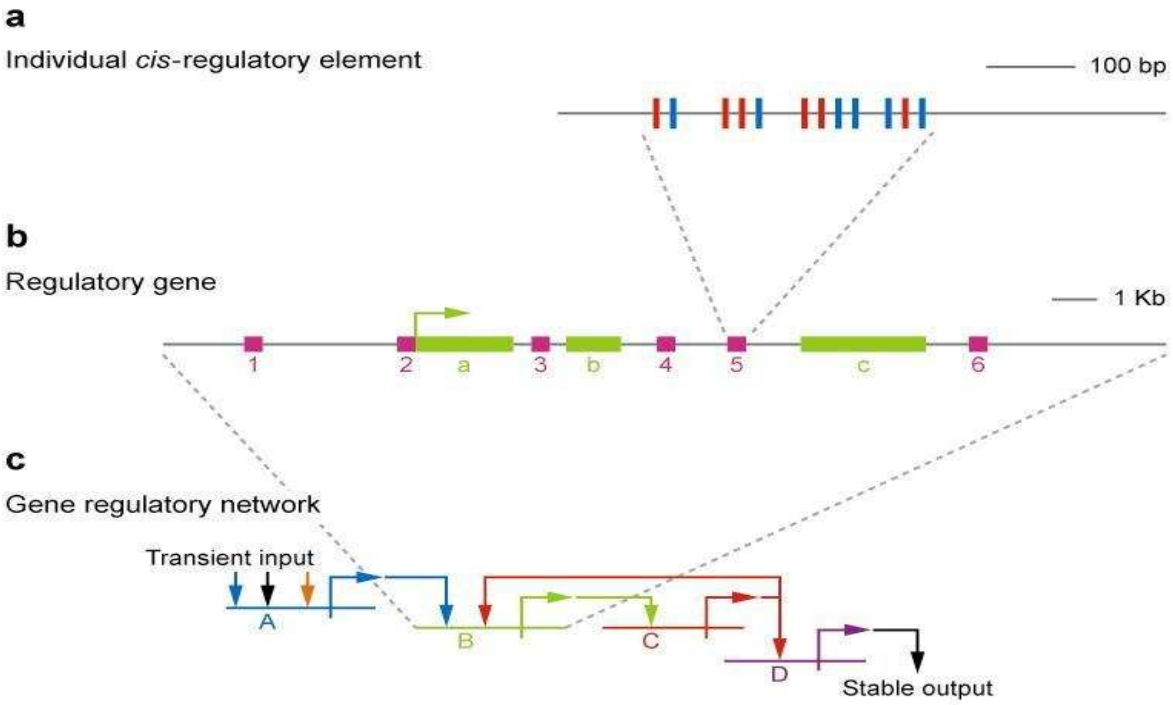


Figure 7: The gene regulatory hierarchy: (*a*) An individual *cis*-regulatory module contains a cluster of several transcription factor binding sites, indicated in red and blue boxes. (*b*) A gene contains a number of *cis*-regulatory modules (*pink boxes*) that control its expression at different times and lineages in the developing embryo. The exons are indicated in light green boxes. (*c*) The inter-regulating transcription factors and signalling molecules form a network that is essentially the genomic program for specification. In this diagram the colour codes of the three levels match, so panel *a* presents the *cis*-regulatory module of gene B (see panels *b* and *c*), which has binding sites of transcription factors A (*blue*) and C (*red*) (Text and figure from Leon and Davidson, 2007).

Transcription regulation is mostly done by enhancers, DNA sequences having multiple binding sites for variety of transcription factors and can activate transcription independent of their location, distance and orientation to that of their target genes' promoters and even for genes located in other chromosomes (Ong and Corces, 2011). A typical gene regulatory region is shown schematically in Figure 8. Silencers are sequences that negatively affect the rate of transcription and share most of the structural features with enhancers (Ogbourne and Antalis, 1998). Insulators or boundary elements limit the transcriptional activity of adjacent region of DNA so as to create pockets of expression domain within the genome (Maston et al., 2006). Locus control regions (LCRs) regulate the transcriptional activity of whole gene cluster in tissue specific and position independent manner and are composed of multiple cis

acting elements including enhancers, silencers and insulators (Q et al., 2002; Maston et al., 2006).

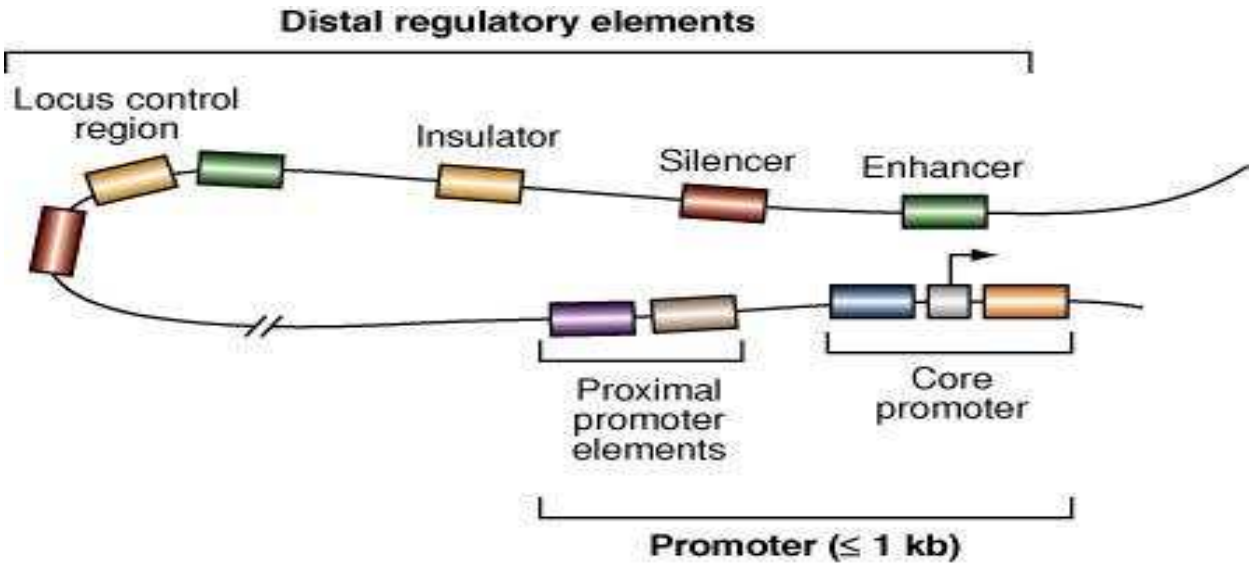


Figure 8: Schematic of a typical gene regulatory region. The promoter, which is composed of a core promoter and proximal promoter elements, typically spans less than 1 kb pairs. Distal (upstream) regulatory elements, which can include enhancers, silencers, insulators, and locus control regions, can be located up to 1 Mb pairs from the promoter. These distal elements may contact the core promoter or proximal promoter through a mechanism that involves looping out the intervening DNA (Text and Figure form Maston *et al.*, 2006).

Enhancers have been shown to carry epigenetic signatures e.g. specific histone marks that are usually established early in development and keep changing as cells differentiate hence modulating gene expression with and without non-coding RNAs (Ong and Corces, 2011). As already detailed in section 1.3.2 that distinct histone modification act sequentially or in combination to give specific downstream signals according to histone code hypothesis. For example specific histone marks that have been shown to be associated with increased gene activity are H3K4 and H3K36 trimethylation, H3 and H4 acetylation while H3K9 and H3K27 trimethylation, H4K20 di and trimethylation signals the transcriptionally repressed chromatin (Bernstein et al., 2006; Van Leeuwen and Steensel., 2005; Meshorer and Misteli, 2006). These all are the modifications occurring in histone tails, similarly modification in core

histone sequences have also been shown to effect nucleosome structure e.g. phosphorylation of H3T45 particularly in apoptotic cells (Hurd et al., 2009). Further these histone modification mainly methylation and acetylation have been shown to cross talk with DNA methylation (Fuks et al., 2005). Hence a complex and co-ordinated interplay of these epigenetic and genetic control elements coordinates the cellular differentiation during vertebrate development making it irreversible and error free (Meissner, 2010).

It has been now well established that Sox2, an endogenous transcription factor in combination with Oct3/4 and Nanog, maintains the differentiation potential of embryonic stem cells, and forced expression of these factors can even reprogram somatic genomes to undifferentiated state (Takahashi et al., 2006; 2007; Park et al., 2008). As described above, development and differentiation are achieved by a precise and tight control of gene expression, it is therefore very important to understand the regulatory mechanisms being used by cells to control expression of these transcription factors and how they become altered during the course of differentiation. The aim of this study was to gain the deeper understanding of the role of different epigenetic regulatory mechanisms during differentiation of mouse embryonic stem cells. The work presented in this thesis has mainly focused on DNA methylation of regulatory regions of Sox2 in mouse embryonic stem cells and a transformed mesenchymal stem cell line to determine whether DNA methylation patterns at these regions could play any role in maintaining differentiation potential or in regulating their differentiation.

1.5 Sox2

Sox proteins have been implicated as crucial players of embryonic development mainly due to their regulatory roles in cellular differentiation pathways hence influencing cell specification by functioning as both transcriptional factors and architectural component of chromatin (Pevny and Lovell-Badge, 1997). They initially came under investigation by identification of their DNA binding domain highly homologous to the Sry box – a sex determining factor located on Y-chromosome in mouse and human (Sinclair et al., 1990; Gubbay et al., 1990). This Sry box encodes a 70-80 amino acid motif highly similar to DNA-binding domain of HMG (high mobility group proteins – so called because of their high electrophoretic mobility due to their small molecular mass <30KDa) box superfamily and hence named Sox (Laudet et al., 1993). HMG box proteins are an extended family of proteins and classified in to two subgroups based on their DNA-binding sequence specificity and number of HMG domains present within a single protein (Laudet et al., 1993). The Sox family of proteins is categorized as having a HMG-box sharing at least 50% sequence similarity with mouse Sry and at present consists of 20 different proteins which are grouped together as subfamilies based on homology within and outside the HMG-box of same family members (Bowles et al., 2000; Schepers et al., 2002).

The HMG domain of Sox factors mediumtes their binding with their target DNA in a sequence specific manner which is unique to Sox proteins and the consensus sequence for them has been identified as the heptameric sequence 5'-(A/T)(A/T)CAA(A/T)G-3' (Harley et al., 1994). The structure of Sry-related HMG domain has been reported both with and without DNA binding and consists of three alpha helices arranged in twisted L-shape whereby helix I and helix II forming a long arm in an antiparallel arrangement and helix III

forms the short arm of L-shape in association with N-terminal extension (van Houte et al., 1995; Werner et al., 1995). The overall structure is maintained by a hydrophobic core whose constituent amino acids are highly conserved among Sox proteins as base specific DNA contacts. HMG domain conformation remains unaffected after binding with target DNA, however a large conformational change is induced into the target DNA structure forcing minor groove towards the concave binding domain of HMG and introducing an overall 70-85˚ bend thus helically unwinding it (Wegner, 1999; Ferrari et al., 1992). The binding in to the minor groove of DNA by Sox proteins is sterically more feasible in its close proximity and is distinct from other transcription factors which bind in the major groove of DNA (van de Watering et al., 1993). This feature of Sox proteins in combination with their ability to bend DNA has led to the speculation that they also serve as architectural component by organizing chromatin structure and assembling other DNA bound transcription factors in to biologically active and sterically defined multiprotien complexes (Wegner, 1999; Werner and Burley, 1997; Wolffe, 1994).

It has been shown by Sox binding sites analysis in the regulatory regions of target genes identified for Sox2 and Sox9 that a partner protein is required to bind with a nearby sequence to enable Sox proteins to act (Kamachi et al., 1995, 2000; Pevny and Lovell-Badge, 1997). Figure 8 illustrates the general structure of a Sox protein and how their binding to their target DNA is stabilized when the target DNA is bound by an additional protein factor able to interact with Sox protein as well.

Sox2 is a transcription factor belonging to the Sox family of proteins described above. Sox2 expression is subjected to both temporal and spatial regulation in mouse: it is first expressed throughout the inner cell mass and later on its expression is restricted to primitive ectoderm,

developing CNS, the lens and primordial gut (Collingnon et al., 1996; Que et al., 2007; Wiebe et al., 2000). Murine Sox2 maps to 3 A2-B locus and is an intronless gene of 2.4 Kb encoding a 319 amino acids protein which shares an overall 98% similarity with human SOX2 (Collington et al., 1996). It belongs to Group B1 of Sox proteins – a subgroup of Group B which is broadly considered as activating members of Sox gene family (Uchikawa et al., 1999).

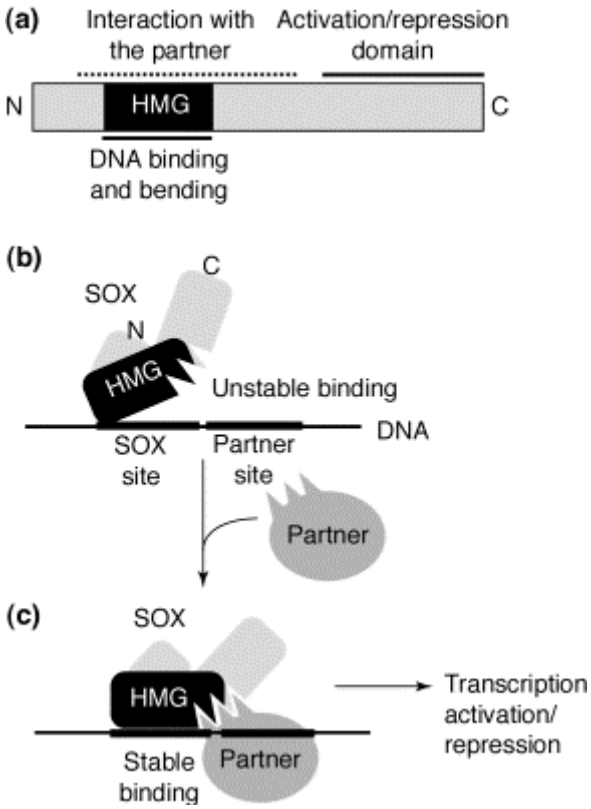


Figure 8: Sox proteins bind with their target stably only in presence of co-factor (Kamachi *et al.*, 2000)

1.5.1 Sox2 – targets

A lens specific gene δ-crystallin was the first described target for Sox2 in chicken which gets activated upon binding with Sox2 in its minimal enhancer element DC5 (Kamachi et al., 1995; 1998). Two important genes for maintaining the pluripotent state of embryonic stem

cells i.e. fibroblast growth factor 4(FGF-4) and undifferentiated embryonic cell transcription factor 1 (UTF1), were later shown to be activated by Sox2/Oct-3/4 complex (Yuan et al., 1995; Ambrosseti et al., 1997; Nishimoto et al., 1999). Now many gene networks targeted by Sox2 alone or in complex with other core transcriptional regulatory circuitry members have been reported (Avillio et al., 2003; Boyer et al., 2005; 2007). Sox2 has also been reported to act as transcriptional repressor, for example as observed at the osteopontin gene (Botquin et al., 1998). It is therefore possible that activating or repressive activity of Sox2 at different regulatory regions is dependent on its surrounding sequences which might recruit different partners. Also depending on which protein it is interacting in specific differentiation pathway, it can act as either activator or repressor.

1.5.2 Sox2 – functions

Different roles have been ascribed to Sox2 so far by different research studies, including maintenance of pluripotent state of embryonic stem cells, specification and maintenance of neural stem cell identity, induction of lens fibre cell differentiation, regulation of various anterior pituitary hormones and taste bud development by endodermal progenitor cell differentiation (Lefebvre et al., 2007).

1.5.3 Sox2 – regulation

Identification of regulatory regions in the genome is quite a complex endeavour given the redundancy of consensus sequence of TFs, compactness of chromatin, concentration of TFs in cell at the given moment, competitive or synergistic binding of TFs to the same or neighbouring region and hence leading to transcription up regulation or down regulation (Pan

et al., 2010). A common method for the study of gene regulation is to first use computational approaches to identify TF binding sites and then to validate them experimentally (Kolchanov et al., 2007). Phylogenetic foot printing is an extensively used approach for the prediction of regulatory sites. Evolutionary conserved non-coding sequence elements of single gene found through cross-species alignment of homologues sequences are thought to be involved in regulation (Pavesi et al., 2007). Affinity data, conformational and physic- chemical properties of TF in complex with its regulatory sequences aid in refining the target prediction (Pan et al., 2010). ECR browser (Ovcharenko et al., 2004) is an extensively used tool to compare genomes (available at http://ecrbrowser.dcode.org/) and Figure 9 is a screen shot showing Sox2 DNA sequence across different vertebrates in coding regions (blue and brown), upstream and downstream non-coding elements (red).

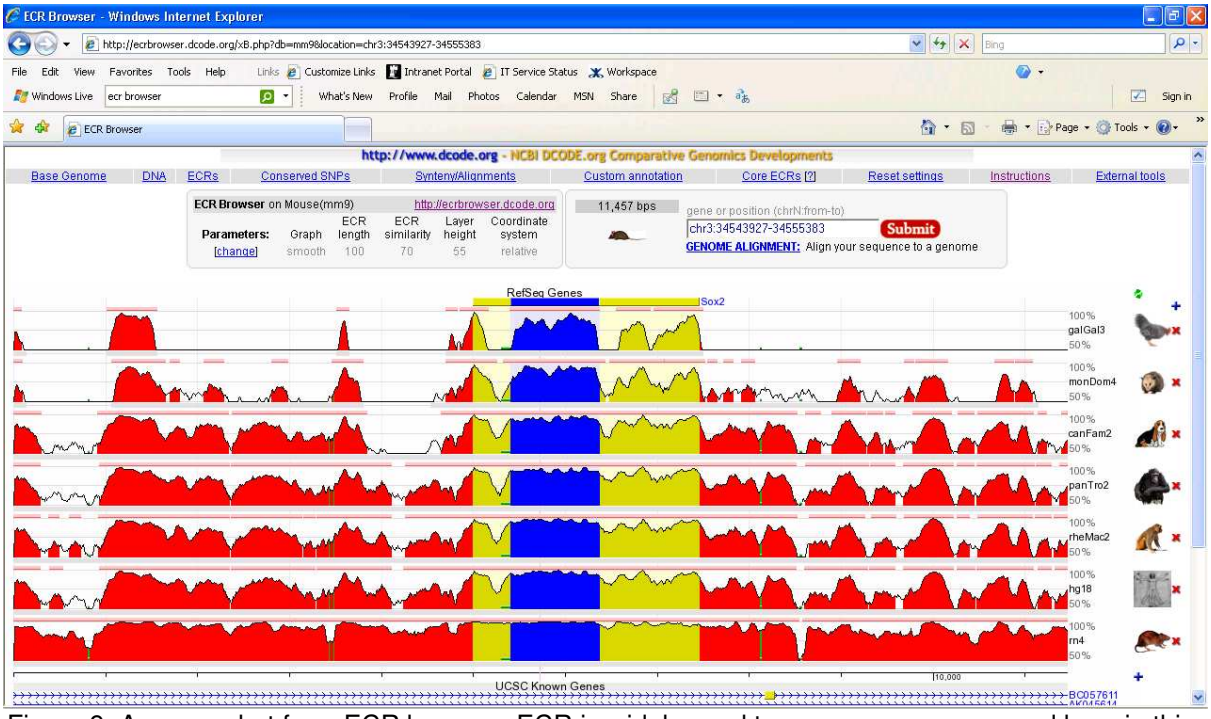


Figure 9: A screenshot from ECR browser. ECR is widely used to compare genomes and here in this screen shot genome sequences coding for Sox2 gene are compared across different vertebrates. Blue and yellow regions indicate the coding sequences of the gene and red regions are non-coding elements found upstream and downstream of the coding region. SRR1 and SRR2 regulatory regions of Sox2 gene are present about 4Kb upstream and downstream of the transcription start site and have been shown to be evolutionary conserved among different species as shown here by genome comparison.

The Sox2 gene has been reported to contain several regulatory regions i.e. core promoter (Wiebe et al., 2000), an ambiguous 5' flanking region (Zappone et al., 2000), and the evolutionary conserved upstream and downstream (~4kb from transcription start sites) enhancers which are designated Sox Regulatory Region 1 and 2 (SRR1 and SRR2) in mouse and humans (Tomioka et al., 2002; Sikorska et al., 2008). SRR1 contains binding site for POU transcription factor and direct neural-specific Sox2 expression while SRR2 binds Oct4-Sox2 and is essential for Sox2 expression both in ES cells and neural stem cells. These enhancers have been shown to exhibit differential DNA methylation and histone H3 acetylation during human neural progenitor's differentiation in astrocytes and neurons making Sox2 permanently or transiently silenced (Sikorska et al., 2008). It is therefore feasible to propose that regulatory mechanisms might have been conserved in mouse and humans, making mouse embryonic stem cells a good model system to study regulation of Sox2 during in vitro differentiation.

1.6. Aims of the research

This study was aimed to understand the molecular regulation of endogenous Sox2 in mouse embryonic stem cells. As described above, Sox2 is a key transcription factor required for maintenance of stem cell like state i.e. their ability to self-renew whilst retaining their differentiation potential. The major focus was on the analysis of DNA methylation of two regulatory regions of Sox2 namely SRR1 and SRR2, in undifferentiated and differentiated mouse embryonic stem cells and mesenchymal stem cells (a transformed cell line). These regulatory regions of Sox2 were analysed at different time points following transition of embryonic stem cells from an undifferentiated state to differentiated state to identify potential onset of DNA methylation changes and how this is correlated with cellular decision making.

In order to determine whether potential differences in methylation were specific to individual lineages and if different level of regulation were observed as cells differentiated towards a lineage in which Sox2 expression is required, similar analyses were performed in mouse ES cells as they differentiate towards the osteogenic lineage (Chapter 3) or neural lineage (Chapter 4).

Bone marrow derived MSCs are the physiological precursors of osteoblasts. MSCs are shown to be readily differentiated in vitro towards the osteogenic lineage and they are currently in use in the clinic. As the protocol used for osteogenic differentiation of MSCs and ES cells in this study are similar, it was thought possible to compare and contrast DNA methylation changes occurring at this gene locus in embryonic and adult stem cell types. It was thus hoped that further insights could be gained on whether there are conserved gene regulatory differentiation systems, among different kinds of stem cells.

Chapter 2 - Methods and Materials

2. Methods and Materials

2.1. Chemicals, reagents and medium

All medium, chemicals and reagent used in this study are listed in Appendix 7.1 detailing suppliers and catalogue numbers. The composition of various medium, solutions and reagents prepared for use in experiments are detailed in Appendix 7.2.

2.2. Cell Culture

2.2.1. Mouse ES cell culture

A frozen aliquot of mouse embryonic stem cells - E14Tg2a cell line created from mouse strain 129/Ola (Hooper et al., 1987) was taken out from liquid nitrogen and defrosted in water bath at 37˚C and quickly resuspended in complete mES medium. Cells were centrifuged at 180g for 5 minutes and during this time a T-25 cell culture flask was gelatinized (0.1% (v/v) gelatine in 1X PBS). Cells were collected after centrifugation, resuspended in 5ml medium and plated in gelatinized flask and incubated at 37˚C in 5% CO_2. Medium was changed the next day and cells were passaged when cells had reached to about 70-80% confluence or after 48 hours at the latest.

For passaging of mES, medium was aspirated and cells washed with 1X PBS. 0.5ml of 0.25% trypsin-EDTA (TE) added per T-25 flask and incubated at 37˚C for 4 minutes. TE activity was inhibited by addition of complete medium in cells which were then passed through 19G needle for no more than four times to disaggregate cell clumps. An aliquot of cell suspension

was taken (about 20µl) to count the cells. Meanwhile, cells were centrifuged and then resuspeneded in complete ES medium at a concentration of 1×10^6 cells per ml. 1ml of cell suspension again seeded in to a new gelatinized T-25 in 5ml of ES medium and cells incubated to grow. This passaging of ES cells continued to get enough cells to set up osteogenic and neural differentiation besides making some aliquots to freeze cells in liquid nitrogen.

For freezing mES cells, cells were trypsinized, passed through needle, counted and resuspeneded in freezing medium at a concentration of 4×10^6 cells/ml and quickly transferred to Styrofoam box containing many layers of tissue to protect cells from temperature shock and stored in -80˚C freezer. After two or three days, cells were transferred to liquid nitrogen.

2.2.2. Embryo Body formation

In order to differentiate mES cells towards the osteogneic and neural lineages, first mES were grown as embryo bodies (EB) in suspension in non-adherent bacterial culture dishes. For this purpose, cells growing in adherent monolayer were trypsinized and counted. 4×10^6 cells were seeded in to 15ml EB medium (medium used was of same composition as complete ES medium except LIF) in non-adherent bacterial culture dishes (90mm) and incubated at 37˚C in 5% CO_2 for three days. Bacterial dishes were occasionally shaken during EB formation to prevent their attachment with surface.

After three days EBs was disaggregated by trypsinization to set up osteogenic differentiation (section 2.2.3). For neural differentiation some of the petri dishes were allowed to remain in suspension for an additional day, and at day four all-trans-retinoic acid (0.5µM) was added in

the EB medium and incubated for a further four days to induce neural precursors formation for setting up neural differentiation (section 2.24).

2.2.3. Osteogenic Differentiation

EBs were collected in 50ml falcon tube after three days of incubation in suspension and trypsinized. First EBs were collected after centrifugation, medium aspirated and added 2ml of trypsin-EDTA and incubated at 37˚C in water bath. EB medium was then added to inhibit TE activity and cells passed through syringe to ensure single cell population. An aliquot was removed for counting and cells were collected by centrifugation at 180g for 5 minutes. Cells obtained after dissociation of EBs were plated in osteogenic medium (differentiation medium) at a concentration of 5×10^5 cells in 3ml of medium per well of six-well tissue culture plate. Four plates were seeded to harvest cells at different time points for gene expression studies, DNA methylation analysis, alizarin red staining and quantification and immunofluorescence for protein markers i.e. 7, 14, 21 and 28 days respectively.

Similarly cells were seeded in control medium which lacks additional factors responsible for osteogneic induction i.e. dexamethasone, betaglycerol phosphate and ascorbic acid. All the controls and osteogenic treated cells were incubated in 5% CO_2 at 37˚C and medium regularly changed every two days. Cells were harvested at day 7, 14, 21 and 28 days of differentiation for RNA and DNA extraction. Also two well of cells were fixed with 4% parafomaldeyde (w/v) for alizarin red staining and DNA quantification and one well for immunofluorescence staining (section 2.7.2) having sterile cover slips placed in them.

2.2.4. Neural Differentiation

At day eight EBs were collected, disaggregated and counted as described above for osteogenic differentiation (2.2.3). 4×10^6 cells were plated in 3ml of N2/F12 medium per well of six-well tissue culture plate. Medium was changed after 2hrs, 24 hrs and 48 hrs from plating. Then N2 medium was replaced with neurobasal medium supplemented with B27 and cells were grown for 28 days, harvesting samples at day 12, 21 and 28 for RNA, DNA extraction and immunofluorescence staining (section 2.7.2).

2.2.5. Mouse MSC culture and osteogenic differentiation

D1 – a mouse mesenchymal stem cells line (Diduch et al., 1993) was retrieved from liquid nitrogen and defrosted in water bath at 37˚C and quickly resuspended in MSC medium. Cells were centrifuged at 180g for 5 minutes, collected after centrifugation, resuspended in 5ml medium and plated in T25 tissue culture flask and incubated at 37˚C in 5% CO_2. Medium was changed the next day and cells were passaged when reached to about 80-85% confluence.

For passaging of MSCs, same protocol was used as described above for ESC (section 2.2.1) except that cells were not passed through syringe. This passaging of MSCs continued to get enough cells to set up osteogenic differentiation besides making some aliquots to cryopreserve. Then MSCs were plated in four tissue culture plates (six-well plates) in the same way as described above for ESC (section 2.2.3) to set up osteogeinc differentiation and harvest cells at specified time points for RNA, DNA and markers protein analysis.

2.3.Molecular Analysis

2.3.1. Reverse Transcription PCR

2.3.1.1.RNA Extraction

mES cells were collected after trypsinzation, washed with 1X PBS and resuspended in TRI-reagent, 5ml per 10^8 cells. After 5 minutes incubation at room temperature, chloroform (0.2ml per ml of TRI reagent) was added to cell lysate and then centrifuged at 12000g in refrigerated centrifuge at 4˚C for 15 minutes. The supernatant was transferred to a fresh RNase free tubes and RNA grade isopropanol (0.5ml per ml of TRI reagent) added and left at -20˚C for an hour. After centrifugation at 12000g for 30 minutes at 4˚C, the aqueous phase was collected in new tube and RNA pellet was washed with 70% ethanol (v/v) and resuspended in RNA grade water. Quantification was then performed by taking absorbance at 260nm using Nanodrop (Thermo scientific).

After RNA extraction, DNase treatment was done to remove contaminating DNA. 200U of DNase added to RNA solution (2µg) with 40U of RNasin and incubated at 15˚C for 45 minutes. Then 1:1 mixed phenol-chloroform was added (120µl/100µl of RNA) and centrifuged at 13000g at 4˚C for 5 minutes to remove degraded DNA. Aqueous phase was transferred to a fresh RNA grade tube and two volumes of chloroform added and again centrifuged and supernatant collected in new tube. 3M sodium acetate was added ($1/10^{th}$ volume of supernatant) with 2 volumes of 100% ethanol to precipitate RNA and centrifuged at 13000g for 5 minutes at 4˚C. Supernatant was discarded and pellet washed with 70%

ethanol (v/v), air dried and finally RNA resuspeneded in water and concentration was measured using Nanodrop.

2.3.1.2.Reverse Transcription

RNA extracted from mES cells at different time points during osteogenic and neural differentiation was reverse transcribed to cDNA for gene expression analysis. cDNA was synthesized using Expand RT from Roche according to their protocol with slight modifications with random primers. First 100ng/µl RNA was incubated with random primers for 10 minutes at 70ºC and then after cooling at room temperature reverse transcriptase, RNasin, dNTPs and enzyme buffer were added and left at 42ºC for 1 hour. Reaction was stopped by placing tubes in ice and then stored at -20ºC. This cDNA was then used as template for PCR using different gene specific primers.

2.3.1.3.Primers

Primer pairs were designed using published genome sequences available on NCBI website and later checked for primer dimer formation, hairpin structures, and cross reactivity using Mac Vector software (MacVector Inc.). All primers were being synthesized from Eurofins MWG Operon and then optimized using temperature gradients and altering other PCR parameters sequentially. List of primer pairs and their optimal annealing and amplification conditions is given in Appendix 7.3 and regions of genes being amplified and primer optimization figures are shown in Appendix 7.4.

2.3.1.4.Polymerase Chain Reaction (PCR) on cDNA

PCR was carried out in 15µl reaction volume which contained 1X Thermopol buffer, 250mM dNTPs, 25µM forward and reverse primers and 0.5U/µl Thermopol Taq Polymerase (NEB). PCR cycle used for amplification was: 95˚C for 3 minutes, 40 cycles of 95˚C for 30 seconds, annealing temperature (optimal annealing temperatures vary for different primer pairs, Appendix 3) for 30 seconds, 72˚C for 30 seconds; a final extension step at 72˚C for 5 minutes.

2.3.1.5.Agarose gel electrophoresis

Amplified DNA subsequent to PCR was loaded on to 2% (w/v) agarose gel after mixing with 6X (final concentration 1X) Orange G (0.15% Orange G, 60% glycerol, 60mM EDTA) loading dye. The gel was run at constant voltage based on size of gel tank in 1X TAE (tris-acetate EDTA) buffer. DNA was stained with ethidium bromide (added in agarose solution prior to solidification at a final concentration of 1.5µg/ml) and following electrophoresis visualization and analysis was performed using Gel documentation system of FujiFilms.

2.3.2. Methylation sensitive PCR

2.3.2.1.DNA Extraction

mES cells were collected after trypsinization, washed with 1X PBS and resuspended in 2ml of cell lysis buffer and incubated at 37˚C overnight. DNA was extracted following the cell lysis method (Sambrook et al., 1989). DNA was extracted from the cell lysate by

phenol/chloroform extraction using phase lock tubes (Qiagen). First phase lock tubes were prepared by centrifugation at 1500g for 2 minutes at room temperature. Then cell lysate was loaded onto phase lock tubes and subsequently equal volume of 1:1 phenol-chloroform added and centrifuged at 1500g for 5 minutes. Without removing the aqueous phase, phenol-chloroform extraction was repeated and finally 2 volumes of chloroform added to the cell lysate and centrifuged. The aqueous phase obtained after this step was transferred to a new 15ml falcon tube and DNA precipitated using $1/10^{th}$ volume of 4M NaCl and two volumes of absolute ethanol. Precipitated DNA was washed with 70% ethanol (v/v) and resuspended in appropriate amount of Tris-EDTA (TE) buffer pH 8.0 and stored at -20˚C.

2.3.2.2.Restriction Digest of genomic DNA

1000ng genomic DNA was digested with MspI and HpaII using the buffer supplied in a 50µl total reaction volume with 40Units of enzyme. The digestion was performed at 37˚C and stopped by heat inactivation of enzyme as recommended by supplier. Afterwards samples were cleaned using phenol-choloroform as described in section 2.3.2.1 for DNA extraction with one exception that DNA was precipitated with 3M sodium acetate instead of sodium chloride. Samples were centrifuged at 13000rpm for 30 minutes, supernatant removed and DNA pellets washed with 70% ethanol (v/v) and air dried. DNA pellets were then resuspended in 50µl of deionised water and quantified using Nanodrop. Then 50ng was used for polymerase chain reaction (PCR) or based on quantification of amount of DNA present template volume altered accordingly.

PCR on genomic DNA was carried out as described in section 2.3.1.4 with annealing temperatures chosen according to primer pair being used. Primer pairs used and their optimal conditions are described in Appendix 3.

PCR products were visualized by running on 2% agarose gels (w/v) as described in section 2.3.1.5 and then photographed using FujiFilms (LAS 4000) and quantified using AIDA image analysis software.

2.3.3. Bisulphite Conversion of genomic DNA

EZ DNA Methylation-Gold kit from Zymo research was used for bisulphite conversion of genomic DNA for methylation analysis following their protocol. First CT conversion reagent was prepared by adding 900µl water, 300µl M dilution buffer and 50µl dissolving buffer to solid reagent, all supplied with the kit and mixed at room temperature for 10 minutes to dissolve conversion reagent. Then 130µl of this CT conversion reagent was added to 20µl (1000ng) genomic DNA extracted from differentiation time points and samples were placed in PCR machine using cycling parameters recommended by manufacturer. After bisulphite treatment, samples were loaded on Zymo spin column, washed and desulphonated using reagents and protocol provided with kit. Finally treated DNA was eluted in eppendorf using deionized water and quantified using Nanodrop. This was then used for PCR using primers specific for regulatory regions of Sox2 and amplified product excised from agarose using Qiagen gel extraction kit and reagents following their protocol. After extraction amplified product was quantified using Nanodrop and Sanger-sequencing was outsourced to Source Biosciences by providing samples and appropriate sequencing primers according to their

guidelines. DNA sequences were analysed using Chromas software (Technelysium Pty Ltd.) and methylation analysis was done using BiQ analyser (Bock et al., 2005).

2.4.Biochemical Analysis

2.4.1. Alizarin Red Staining and Quantification

Alizarin red staining was performed on cells after osteogneic differentiation (section 2.2.3) using this method (Gregory et al., 2004). Following fixation with 4% (w/v) Paraformaldehyde (PFA) cells were stored in Phosphate buffer saline (PBS) pH 7.4 at 4˚C. To perform the staining PBS was removed from the fixed monolayers of cells and cells washed three times 5 minutes each with distilled water at room temperature with shaking. After washing enough 40mM alizarin red dye solution (about 1ml) was added to each well of the plate to cover the cells and then left for 20 minutes at room temperature with shaking to develop the colour. Then the unincorporated dye was removed by thoroughly washing wells with water four times with shaking for 5 minutes before taking pictures under the microscope.

For quantification of dye incorporated into the cells 800µl acetic acid (10% v/v) was added to each well and incubated at room temperature while shaking for 30 minutes. After incubation, monolayer was scraped from plates with the help of cell scraper and transferred to 1.5ml eppendorf tube with 10% acetic acid. Tubes were then vigorously vortexed for 10 seconds and heated at 85ºC for 10 minutes. After heating tubes were incubated in ice for 5 minutes to allow them to cool and then centrifugation was done at 15000g for 30 minutes. An aliquot of supernatant (about 500µl) was then taken in new eppendorf tube and pH tested so as to be in

the range of 4.1-4.5. Then 150µl of supernatant was transferred in three wells of a 96 well plate for each time point and absorbance was measured using Tecan (infinite M200) plate reader at 405nm. Standards of alizarin red were prepared in the range of 1000µM to 7.8µM by sequential two fold dilutions and read in the same plate at 405nm to create a standard curve for quantification of alizarin red amount extracted from stained monolayers.

2.4.2. DNA Quantification

In order to normalize the amount of alizarin red dye extracted from cells to cell count, DNA was quantified at each time point using method described here (Rago et al., 1990). PBS was removed form fixed monolayers and plates were subjected to three freeze/thaw cycles at -80ºC for about 30 minutes each. Subsequent to this freeze/thawing, cells were scratched from plates and transferred to 1.5ml eppendorf with TNE buffer and centrifuged at 1000rpm for 5 minutes to get rid of cell debris. An aliquot of 75µl supernatant was then transferred to three wells of a 96 well plate and 75µl of Hoechst stain added. Samples were then read using Tecan plate reader with excitation 360nm/Emission 460nm. DNA standards were prepared using mouse genomic DNA in the range of 10000pg to 78.12pg with two fold dilution in TNE buffer and standard curve generated to quantify DNA.

2.4.3. Immunocytochemistry

To perform immunocytochemistry experiments, cells were seeded on cover slips which were placed in the wells of a six well plate (four cover slips per well) while setting up differentiation (section 2.2.3, 2.2.4. 2.2.5). Cultured cells were fixed on cover slips by addition of 4% (w/v) paraformaldehyde at room temperature and then kept in PBS pH 7.4 at

4ºC. In order to stain the cells for the presence of proteins of interest using fluorescent labelled antibody, cells were washed 10 minutes with PBS containing 0.1% Tween-20 (v/v) with shaking to make them permeable. Cells on cover slips were then blocked using block solution (PBS, 0.1% (v/v) Tween-20, 0.2% (v/v) serum) at room temperature for 1 hour and then incubated with primary antibody diluted in blocking solution over night at 4ºC. Next day cover slips were washed with PBS containing Tween-20 three times for 15 minutes each with shaking and then incubated with secondary antibody for one hour diluted in same block as that of primary antibody in dark at room temperature. From this point on wards, care was taken to minimize exposure to light. After incubation with secondary antibodies, again cover slips were washed with PBS containing Tween-20 three times for 15 minutes each and finally mounted on glass slide using Vectra shield. Cover slides were kept in dark at 4ºC until observation and then cells were visualized using Nikon Eclipse 90i fluorescent microscope and images were taken using Volocity 3D image analysis software (PerkinElmer).

Chapter 3 - DNA methylation analysis of Sox2 regulatory regions in mouse embryonic stem cells (E14Tg2a) cultured in osteoblast differentiation medium

3.1. Introduction

As explained in section 1.2, mouse ES cells are now used as a model to study developmental events and understand molecular regulation of varied differentiation pathways. This study aimed (section 1.6) to understand if DNA methylation, a major epigenetic mechanism of gene regulation, of Sox2 regulatory regions in embryonic stem cells gets altered as cells differentiate to osteogenic and neural lineages derived from germinal ectoderm and mesoderm respectively. This chapter summarizes the findings of experiments about mouse embryonic stem cells grown in medium known to promote differentiation to osteoblasts (bone cells). Neural differentiation results are detailed in chapter 4.

3.1.1. Bone Formation/Osteogenesis

Bone is a specialized connective tissue under constant formation and resorption by osteoblasts and osteoclasts respectively throughout the life span of a vertebrate to maintain bone mass and calcium homeostasis (Aubin and Triffitt, 2002). Bone tissues are made up of hydroxyapatite crystals and an extracellular matrix consisting of type I collagen, osteocalcin, osteopontin, bone sialoprotein and proteoglycans (Young et al., 1992; Robey et al., 1993). Osteoblasts are cells that secrete and deposit most of these proteins, participate in the formation of hydroxyapatite crystals and have been shown to display high alkaline phosphatase activity besides responding to osteotropic hormones and cytokines (Mundlos and Olson, 1997).

In mammals bone tissue has been reported to form through two independent pathways: intramembranous ossification and endochondral ossification. During intramembranous

ossification, osteoblasts differentiate directly from mesenchymal cells (detailed in Figure 3.1).

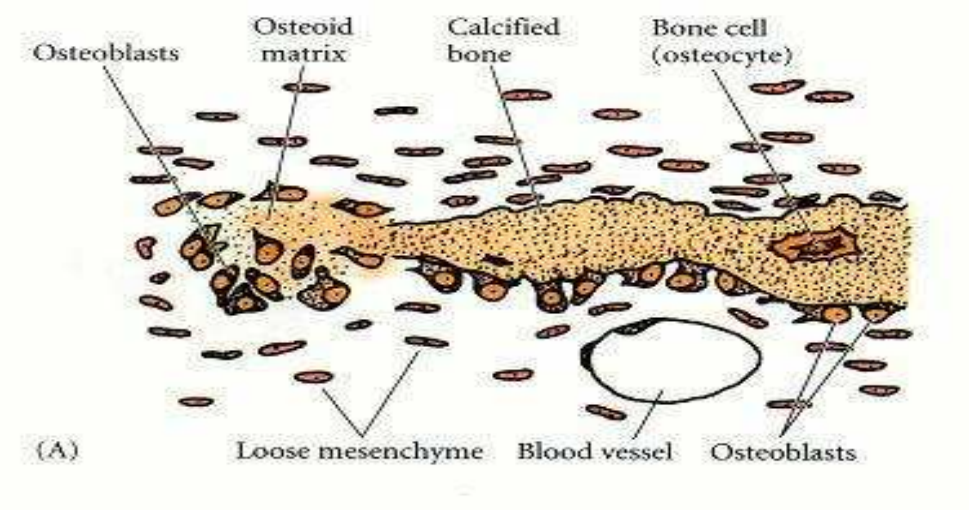


Figure 3.1: Schematic diagram of intramembranous ossification. (A) Mesenchymal cells condense to produce osteoblasts, which deposit osteoid matrix. These osteoblasts become arrayed along the calcified region of the matrix. Osteoblasts that are trapped within the bone matrix become osteocytes. (Text and figure taken from Gilbert, 2000)

In endochondral ossification, chondrocytes arise first from mesenchymal condensations and form a cartilaginous template. This template is subsequently mineralized by osteoblasts derived from surrounding mesenchymal stem cells once hypertrophic chondrocytes mature. The matrix around hypertrophic chondrocytes becomes calcified and invaded by blood vessels (shown in Figure 3.2). Bone marrows forms after the deposition of a bone matrix and then osteoclasts differentiation proceeds (Karsenty, 1999).

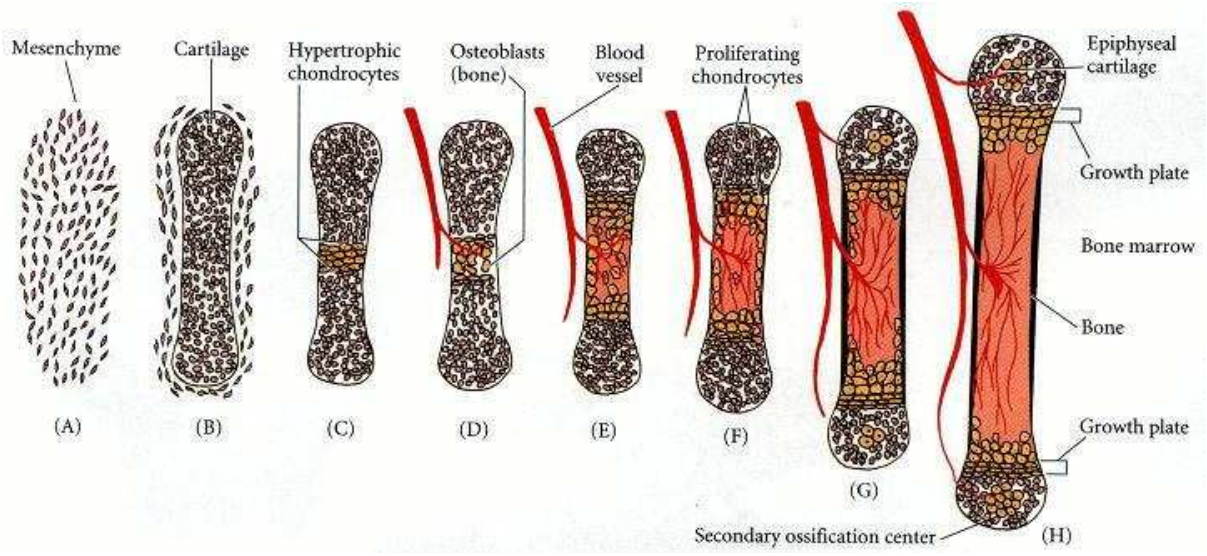


Figure 3.2: Schematic diagram of endochondral ossification. (A, B) Mesenchymal cells condense and differentiate into chondrocytes to form the cartilaginous model of the bone. (C) Chondrocytes in the center of the shaft undergo hypertrophy and apoptosis while they change and mineralize their extracellular matrix. Their deaths allow blood vessels to enter. (D, E) Blood vessels bring in osteoblasts, which bind to the degenerating cartilaginous matrix and deposit bone matrix. (F-H) Bone formation and growth consist of ordered arrays of proliferating, hypertrophic, and mineralizing chondrocytes. Secondary ossification centers also form as blood vessels enter near the tips of the bone. (Figure and its description are taken from Gilbert, 2000)

Mesenchymal stem cells or stromal stem cells are physiological precursors of osteoblasts and by definition are bone marrow derived fibroblasts that can differentiate in osteoblastic, adipogenic and chondrocytic lineages given the suitable stimuli (Pittenger et al., 1999). The process of bone formation is under complex regulatory control of myriad of signalling cascades such as fibroblast growth factors (FGFs), bone morphogenic protiens (BMPs), Wnt, Notch and, transcription factors but the exact molecular framework of genetic regulation is still elusive (Kozhevnokova et al., 2008; Karsentay et al., 2009).

Some of the key transcription factors regulating conversion of mesenchymal stem cells to mature osteoblasts and their interactions with each other are shown in Figure 3.3.

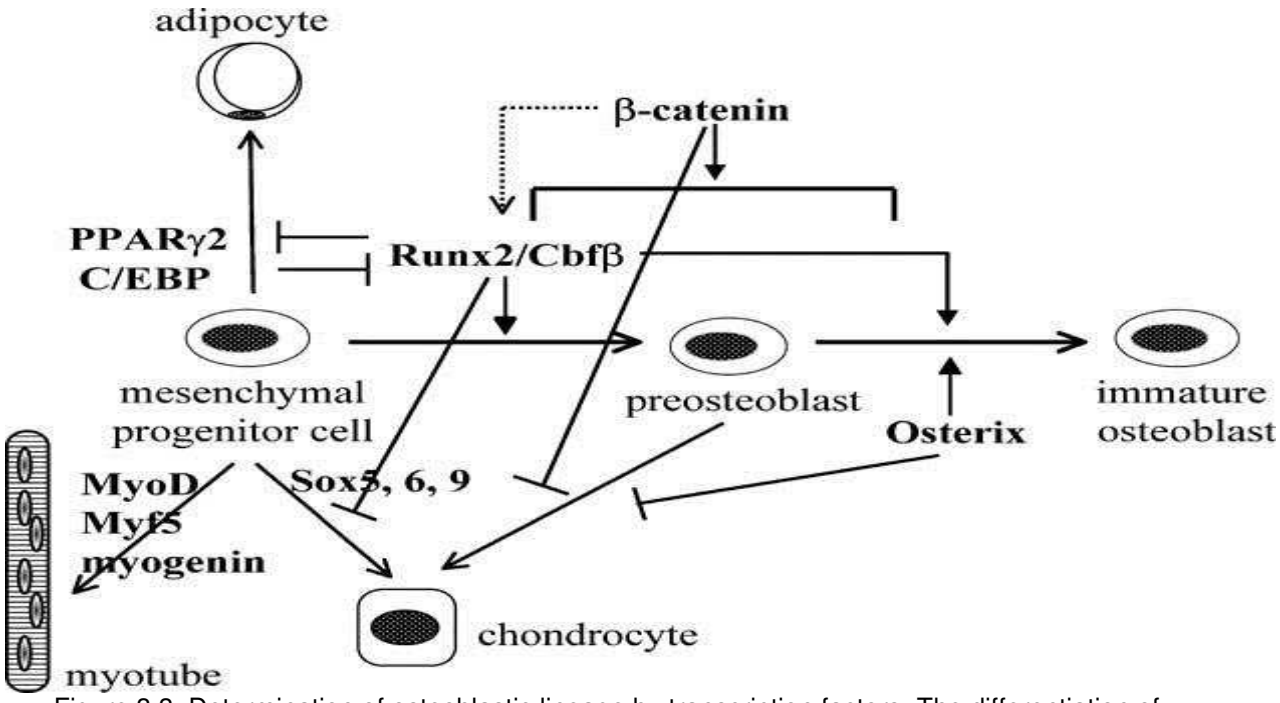


Figure 3.3: Determination of osteoblastic lineage by transcription factors. The differentiation of common mesenchymal progenitor cells into each skeletal component cells is determined by different transcription factors. In osteoblast differentiation, Runx2 directs mesenchymal progenitor cells to preosteoblasts, inhibiting adipocyte and chondrocyte differentiation. Runx2, β-catenin, and Osterix direct preosteoblasts to immature osteoblasts that express bone matrix protein genes and completely eliminating the potential to differentiate into chondrocytes (Text and figure from Komori, 2006).

3.1.2. ES cells as a model to study osteogenesis

Musculoskeletal and osteodegenerative disorders have been well documented as major sufferings of vast majority of aging population but still effective therapies to a large extent are not available (Alfred et al., 2010). Embryonic stem cells are harvested from inner cell mass of embryo and have been shown to develop into any cell type in vitro making them as a possible source for transplantation and other clinical applications (Smith, 1998). The ability of embryonic stem cells to renew themselves indefinitely and still retaining the multilineage differentiation potential gives them a competitive advantage over other stem cell types and hence can potentially serve as a limitless supply of any cell type.

Directed differentiation of ES cells to osteoblasts has been successfully attempted so far in either of two major ways: use of three dimensional spherical structures called embryoid bodies (EB) prior to culturing single cells in a defined culture milieu known to induce osteogensis, and direct culture of dissociated ES cells in conditioned medium avoiding EB formation (Hwang et al., 2008). Embryoid bodies are 3D near spherical structures containing cells of all three germinal layers namely ectoderm, mesoderm and endoderm and recapitulate the environment of early embryonic development (Itskovitz-Eldor et al., 2000).

The major limitation in EB-mediated differentiation strategies is poor characterization of molecular and signalling networks governing the cell fate decisions and hence spontaneous differentiation to unwanted cell types may lower the efficiency in terms of yield and homogeneity (Hwang et al., 2008). Co-culture and conditioned medium can be employed to overcome this bottleneck but then the risk of pathogen transmission and undefined composition of medium limits the use of this approach in clinical setting (Heng et al., 2004). Also this can be more expensive than EB formation. Another strategy that has also been reported is through genetic manipulation for expression of some transcription factors known to induce osteogenesis such as osterix but that also involves potential risks associated with recombinant approaches if differentiated cells have to be used for treatment (Tai et al., 2004).

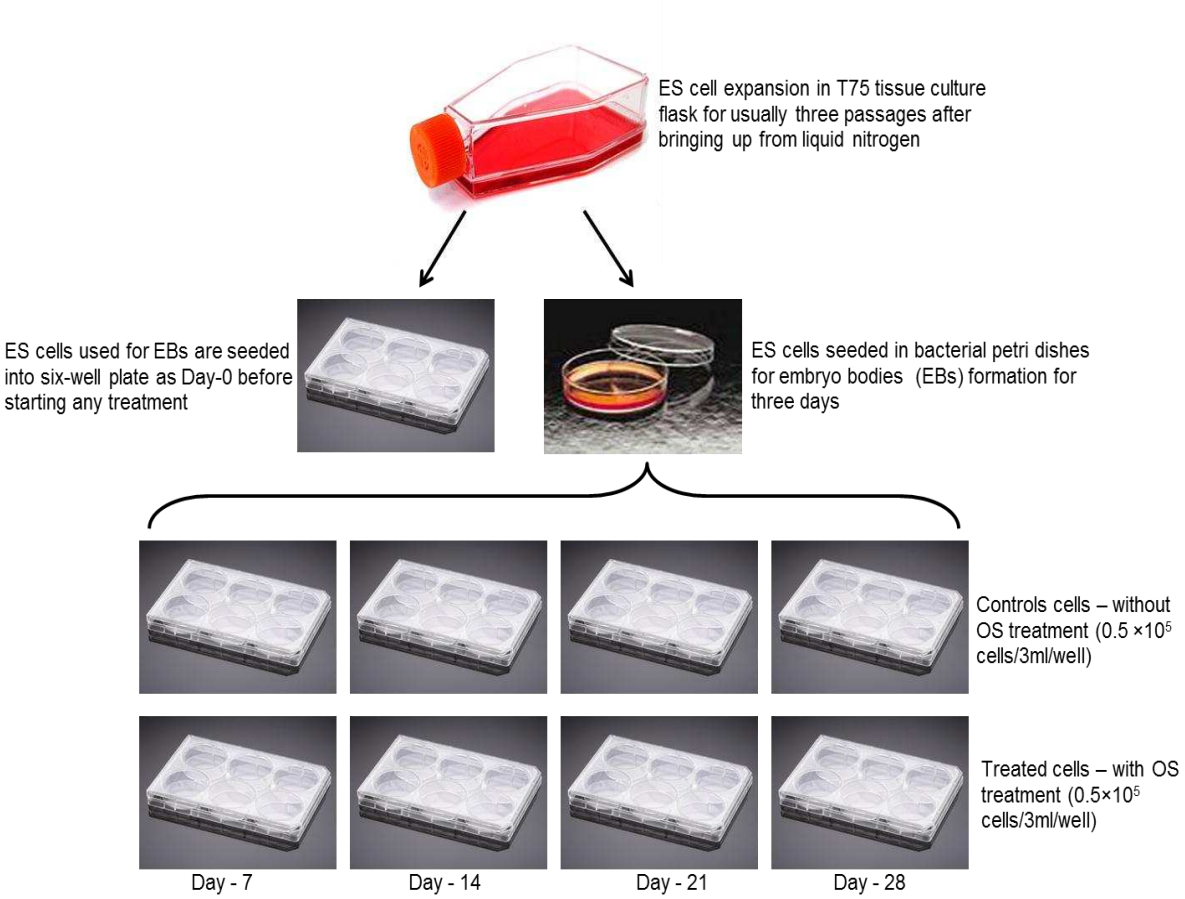
3.1.3. Sox2 in Osteogenesis

There are only limited studies so far investigating the role of Sox2 in osteoblast lineage in particular. Sox2 has been shown to be expressed at low levels in osteoprogenitors and induced by FGF signalling and, favours their proliferation potential over terminal differentiation to osteoblasts by activating Wnt signalling pathway (Ambrosetti et al., 2008; Mansukhani et al.,

2005). Some recent studies have shown experimentally that Sox2 is required for self-renewal of osteoblast precursors and inhibit their differentiation (Roy et al., 2010).

In order to identify the mechanisms that could possibly contribute to down regulation of Sox2 in mature osteoblasts, two regulatory regions namely SRR1 and SRR2 were analysed for DNA methylation changes in this study. This is the first study looking at DNA methylation changes of Sox2 regulatory regions in osteoblast lineage. It was hoped that this information would help to better understand the regulatory role of Sox2 in osteogenic differentiation. Also as described above that MSCs (section 3.1.1) are physiological precursors of osteoblasts so a parallel set of experiments were carried out with MSCs keeping differentiation protocol and other experimental parameters identical to compare and contrast the molecular changes occurring in both cell types. The results of these experiments are detailed in chapter 5.

3.2. A schematic representaiton of experimental work plan



[Cells from each well were then used for subsequent analyses such as Immunocytochemistry (sterile cover slips were placed in the well prior to inoculation of cells, Alizarin red staining and quantification, DNA quantification, RNA and DNA extraction]

3.3.Results

3.3.1. Cell Culture

3.3.1.1.Revival and passage of E14Tg2A cells

E14Tg2A (wild type embryonic stem cells line) were revived from liquid nitrogen and grown on gelatine-coated tissue culture flasks. They were passaged every other day by trypsinization until the majority of the cells in culture looked undifferentiated as described in methods section (2.2.1). The morphology of cells at different passages is shown in Figure 3.4.

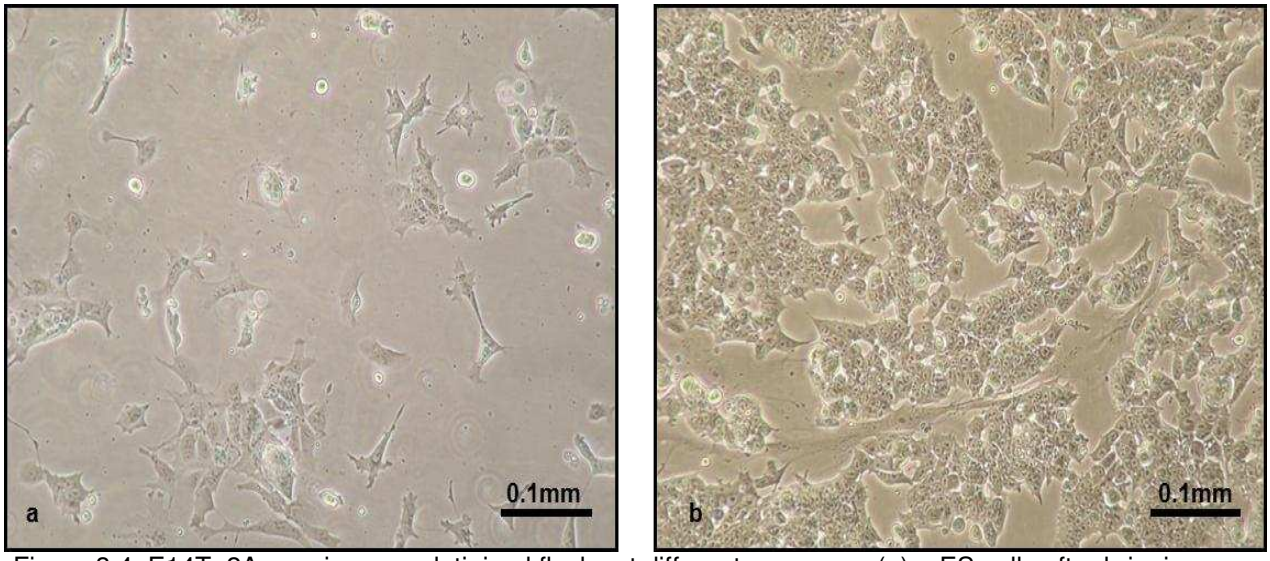


Figure 3.4: E14Tg2A growing on gelatinized flasks at different passages. (a) mES cells after bringing from liquid nitrogen. (b) mES cells with flatten and uniform morphology and then some were cryopreserved and some were used to set up differentiation (all pictures were taken at 10X magnification).

3.3.1.2. In vitro differentiation of mES cells to osteoblasts

ES cells were first grown in gelatinized flasks in undifferentiated state and then allowed to form aggregates, i.e. embroid bodies, in bacterial dishes (Figure 3.5) which were then disaggregated and plated in medium supplemented with osteogenic supplements as described in detail in methods section 2.2.2.

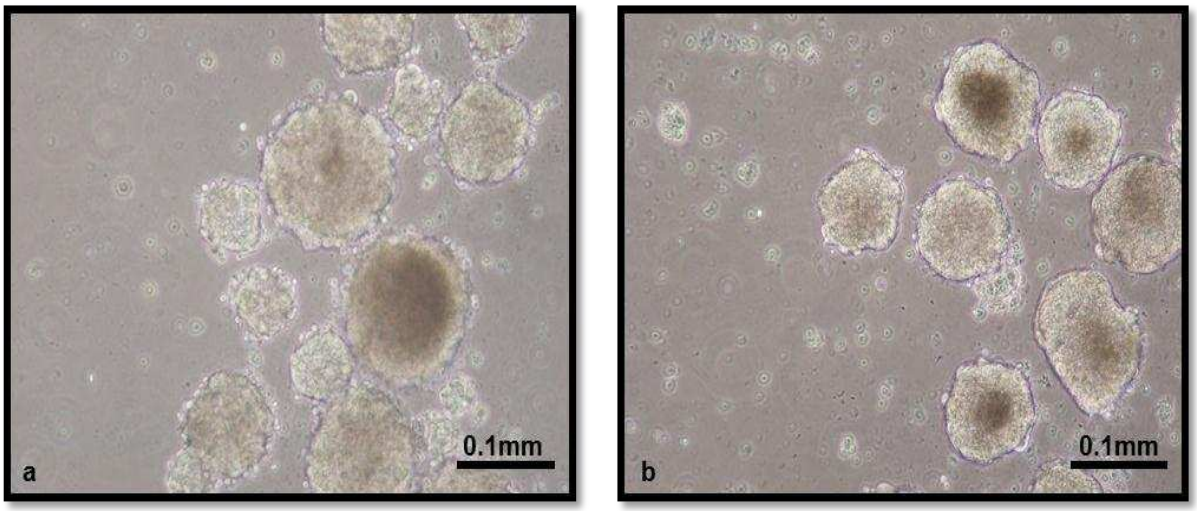


Figure 3.5: (a-b) E14tg2a cells at day-3 in EB medium forming EBs (all pictures were taken at 10X magnification).

Previous research work indicates that embryonic stem cells grown in presence of osteogenic supplements (OS) form colonies with mineral deposition. Mineralization can be observed as a black layer surrounding the cells by phase contrast microscopy when differentiating osteoblasts form calcified matrix and as shown in Figure 3.6 the majority of the cells by day-14 had started to form a dense matrix which was further confirmed by alizarin red staining, stain extraction and quantification. Alizarin red staining has been used historically to demonstrate the presence of calcium-rich deposits in histology for staining bones and skeleton (Puchtler et al., 1969). The staining mechanism is thought to involve absorption of alizarin red molecule on calcium of calcium hydroxyapatite, a major component of calcified

bone by chelate formation (Moriguchi et al., 2003). Use of Alizarin red dye has an added advantage in particular that, it can be later extracted from the entrapped cells and then can be used as a quantitative index to access the extent of mineralization (Gregory et al., 2004).

OS supplements used in medium were ascorbic acid, beta glycerol phosphate and dexamethasone. Beta glycerol phosphate has been reported to act as a source of phosphate ions for in vitro mineralization and ascorbic aicd is required for formation of collagenous matrix (Binderman et al., 1986; Chentoufi et al., 1993). As can be seen in Figure 3.6 and 3.7, the cell line E14tg2a when cultured under these conditions, displays a gradual differentiation towards the osteogenic lineage with increasing density of dark granules that stained red with Alizarin red dye. Cells grown in same medium but lacking osteogenic supplements i.e. beta glycerol phosphate, ascorbic acid and dexamethasone were taken as control and found to be of mixed morphology and to some extent showed mineral deposition towards the end of differentiation experiment which was further confirmed by alizarin red staining (Figure 3.7).

However this also need to be pointed that this medium composition may also lead to nonspecific mineral deposition which may not be cell mediumted and so additional characterization for the presence of matrix associated proteins was also carried out to ascertain whether mineralization is cell mediumted or nonspecific. Osteocalcin as discussed in section 3.4 is one of the major and matrix specific protein in osteoblasts. Gene expression analysis and immunocytochemistry were employed to stain for the expression of osteocalcin at the levels of RNA and protein in differentiating cultures and results are shown in Figures 3.22, 3.20 and 3.21 respectively. As is evident from these figures that expression of osteocalcin both at RNA and protein level coincided well with the pattern of Alizarin red

staining strengthening the observation that cells grown under these culture conditions might be mineralizing.

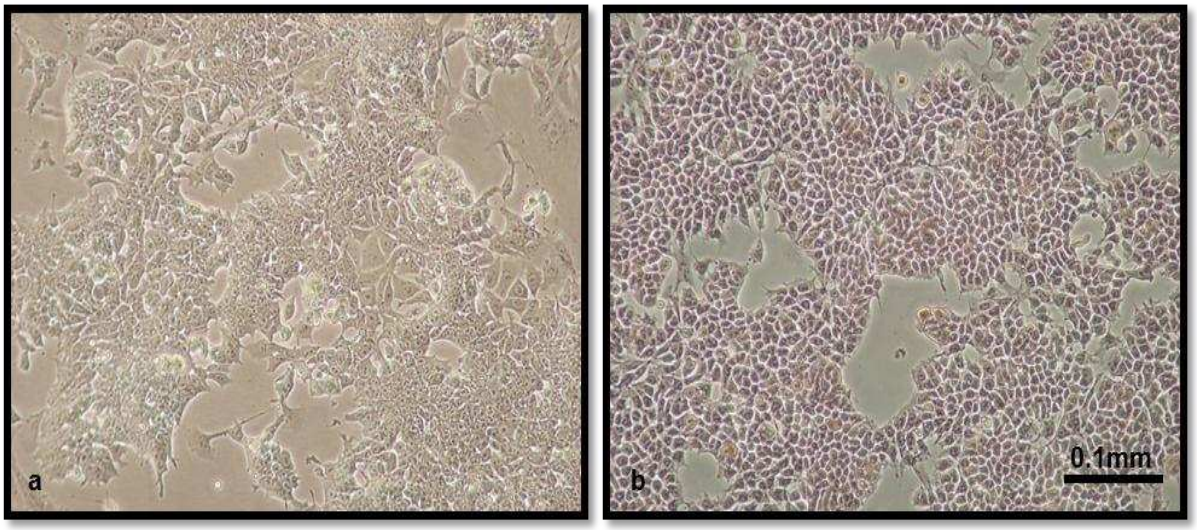


Figure 3.6: E14tg2a cells at day-0 before starting differentiation treatment (a) and stained with alizarin red dye (b) (all pictures were taken at 10X magnification).

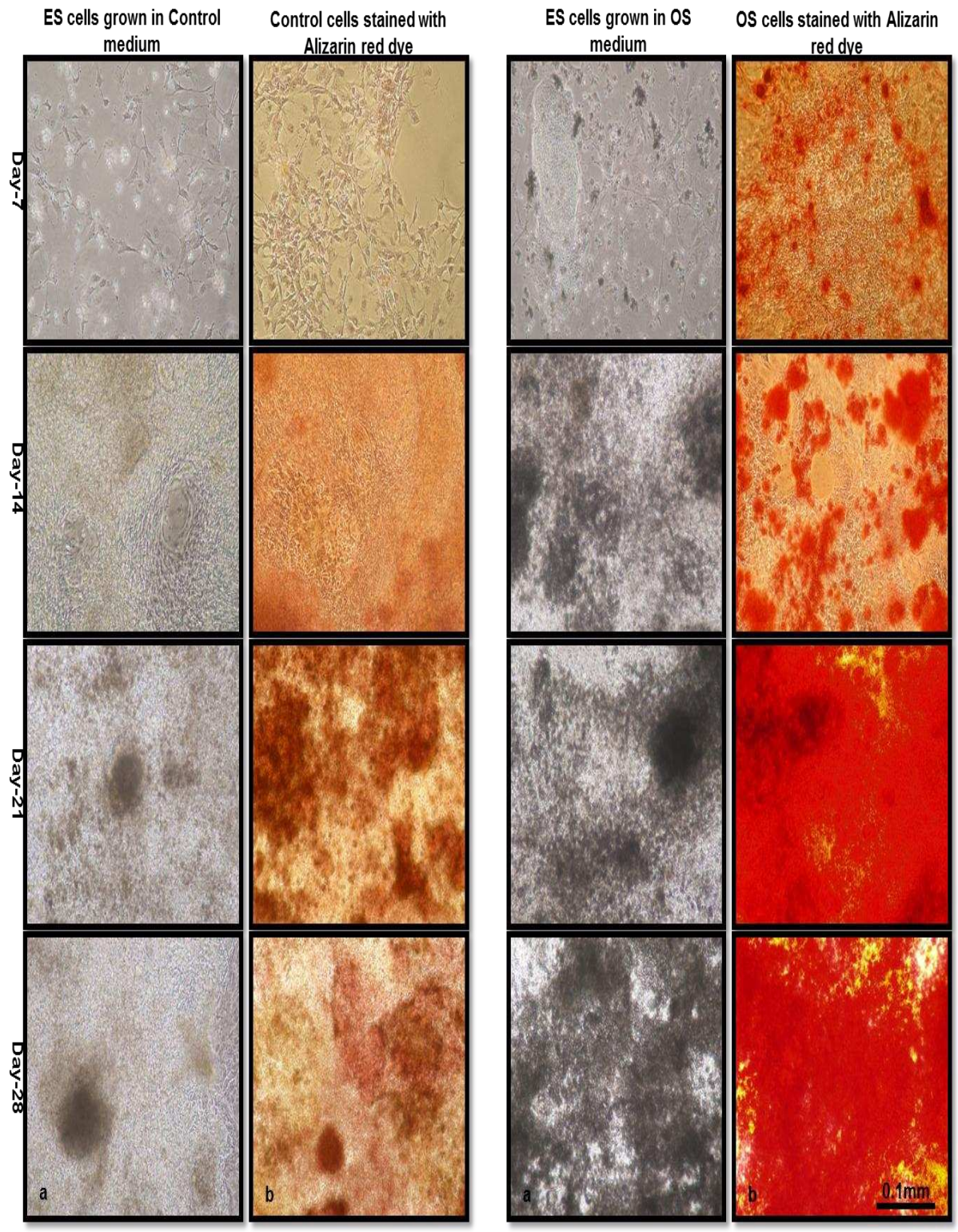


Figure 3.7: E14tg2a cells grown in medium supplemented without and with ascorbic acid, beta glycerol phosphate and dexamethasone i.e. control and OS respectively and analysed by microscopy (a) and alizarin red staining (b). Mineral deposition can be seen as densely dark areas as cells differentiate (a) and they stained dark red with alizarin red (b) (All pictures were taken at 10X magnification). It has been detailed earlier in section 3.3.1.2 that such a staining could also be possible due to non-specific calcium accumulation in culture and hence more data is presented in section 3.3.3 and 3.3.4 to support these results.

3.3.2. Alizarin red dye extraction and quantification

Alizarin red dye was extracted after staining of cells and quantified as described in detail in chapter 2 (section 2.4.1) and normalized to cell count (section 2.4.2). Briefly the amount of alizarin red incorporated by cells after staining was calculated using a standard curve (Figure 3.8). This amount of alizarin red was then normalized to cell count of that well measured by DNA quantification (Figure 3.9). Since technical limitation did not permit to perform both quantification assays using the same well, two wells of six-well plates were seeded at the time of setting up differentiation experiment with equal number of cells and treated alike in every respect except the quantification assay itself.

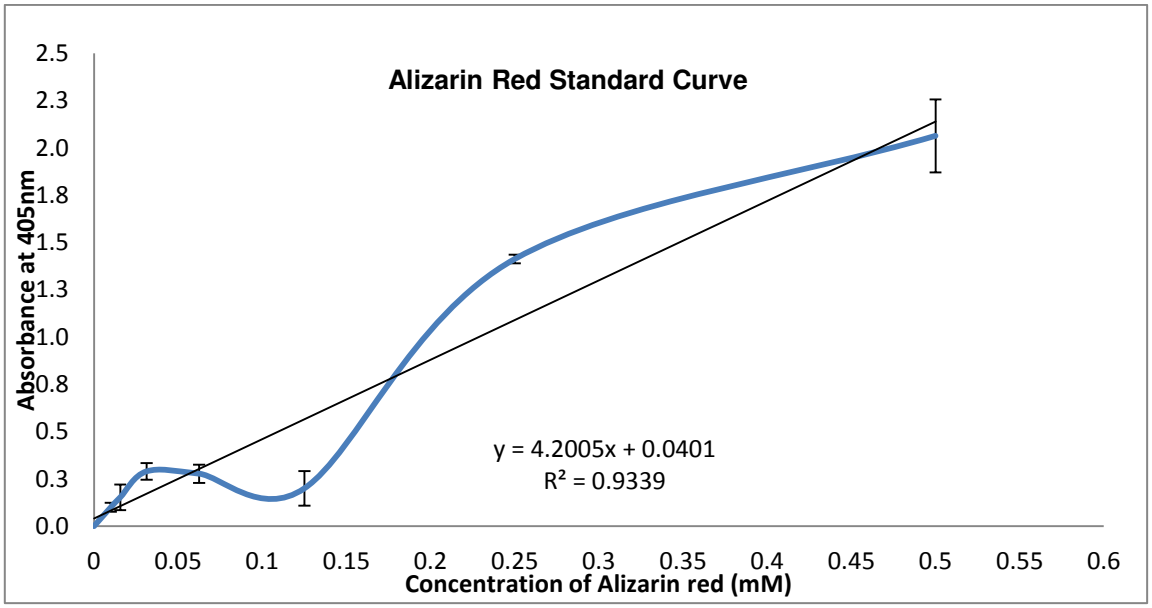


Figure 3.8: Alizarin red standard curve generated to calculate the amount of alizarin red dye extracted from cells (data is collected from two plates and each analysed in triplicates so mean±SEM with n=2)

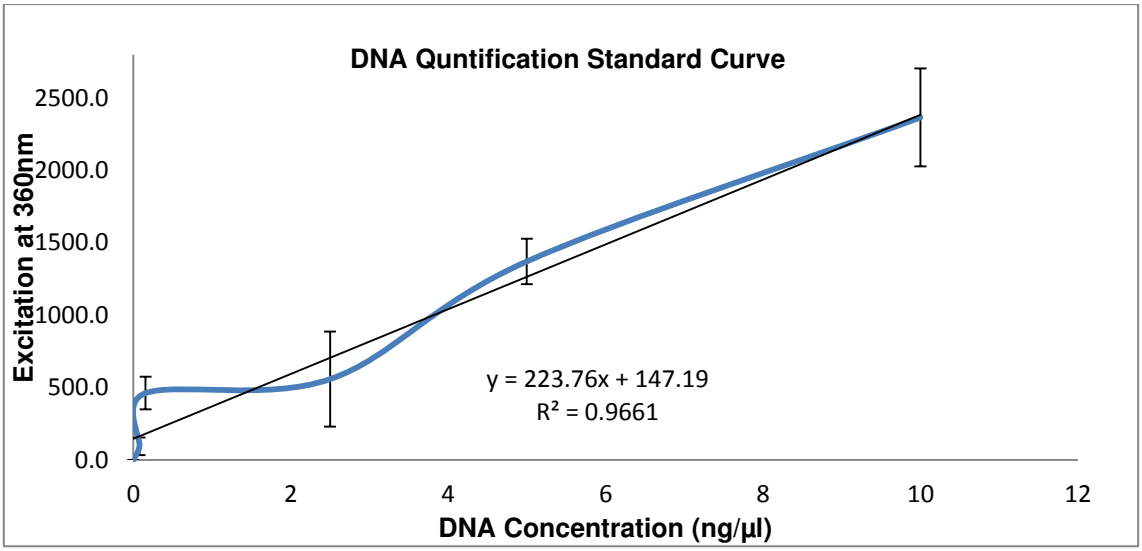


Figure 3.9: DNA calibration curve generated to normalize the amount of alizarin red dye relative to cell contents of plates (data is collected from two plates and each analysed in triplicates so mean±SEM with n=2)

Cells grown in medium containing osteogenic supplements showed a marked increase in amount of alizarin red dye extracted in comparison to cells grown in medium devoid of them after two weeks of culturing as shown in Figure 3.10.

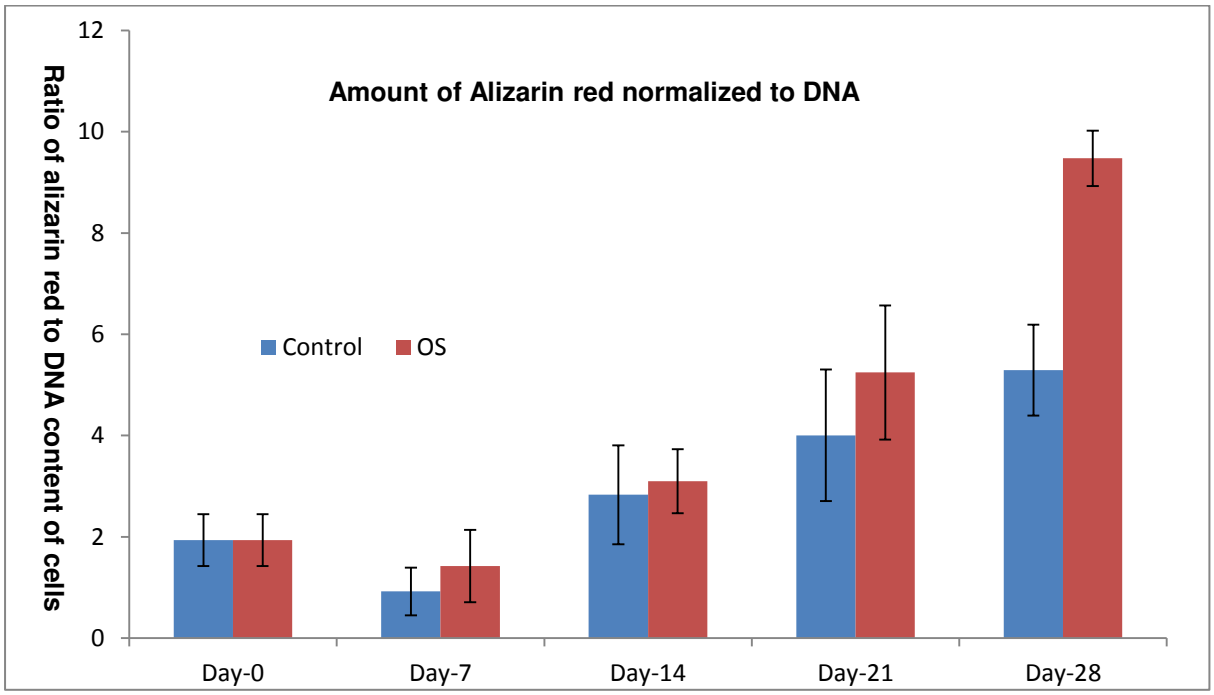


Figure 3.10: Mineral deposition was detected by Alizarin red dye and then dye was acid extracted and quantified using a colourimetric assay. Data are collected from two plates, each analysed in triplicates and expressed as mean±SEM (n=2). There seems to be a marked difference in amount of alizarin red extracted from treated and untreated cells.

It needs to be emphasized here from statistical point of view that this particular assay was carried out twice in triplicates for time-course samples obtained from two independent biological replicates because only one well of 6-well plate was seeded with cells to perform Alizarin red staining when differentiation experiment was set up for the first time. As explained earlier that technically it was not possible to carry out both alizarin red staining and DNA quantification assays using one well from multi-well plate, hence this was corrected in subsequent experiments and data collated and documented here.

3.3.3. Immunocytochemistry

Immunocytochemistry was performed using antibodies against Sox2, Sox9, Osterix and Osteoclacin proteins to detect the localization and expression of these markers (antibody supplier details are in appendix 7.1). Mouse ES cells - E14tg2a (used in this study), mouse NSCs from lateral ventricle (kindly prepared and given by Stephanie Strouhbecker, a fellow student working with Dr Virginie Sottile), primary mouse calvaria and primary mouse osteoblasts cells (kindly given by Omar Qutachi, a fellow student working with Dr Lee Buttery) were used as positive control for Sox2, Sox9, Osterix and Osteocalcin antibodies respectively. And mouse primary calvaria cells were taken as negative control for Sox2 antibody and mouse ES (E14tg2a) were used as negative controls for Sox9, Osterix and osteocalcin respectively (Figures 3.11-3.18). In addition all these cells were also stained with primary antibody only (Figure 3.21), secondary antibody only (Figure 3.21) and no antibody to account for nonspecific and/or background staining(Figure 3.19-3.20).

Almost all of the cells in undifferentiated ESC culture were found to express Sox2 protein. Then only small population of the cells were found to express Sox2 protein at day-7, 14 and

21 in control cells and at day-28 no signal was detected (Figure 3.22). Similarly cells grown in medium containing OS supplements were found to express Sox2 at day-7 and day-14 and then no signal was detected as shown in Figure 3.23.

Sox9 protein expression was not observed in ESCs at day-0. Cells grown in medium lacking OS supplements started to show Sox9 protein expression at day-7 which appeared to persist until day-28 (Figure 3.24). While cells grown in medium containing OS supplements were found to express Sox9 at day-7 which increased with time until day-21 and then fewer cells were found positive a day-28. Some representative fields are shown in Figure 3.25. Sox9 expressing cells were more in number in OS-treated culture than non-treated cells.

Osterix protein was not found to be expressed at any time points chosen for analysis in cells grown with and without OS promoting factors as shown in Figure 3.26 and Figure 3.27.

Osteocalcin was not found to be expressed by ES cells at day-0. Cells were found be expressing osteocalcin starting from day-7 until day-28 grown in both kinds of medium with and without ostoegenic supplements where signal in treated cells was significantly stronger than control population as can be seen in Figure 3.28 and 3.29.

An important consideration regarding all the figures shown on next few pages from Figure 3.11-3.21 is that Day-0 refers to undifferentiated embryonic stem cells (E14tg2a) before subjecting them to any differentiation treatment and is the common experimental starting point before seeding cells derived from EBs into either control medium or OS medium from Day-7 to Day-28 (schematics of experimental design are elaborated in section 3.2).

Controls for Antibodies

3.3.3.1. Sox2 Positive

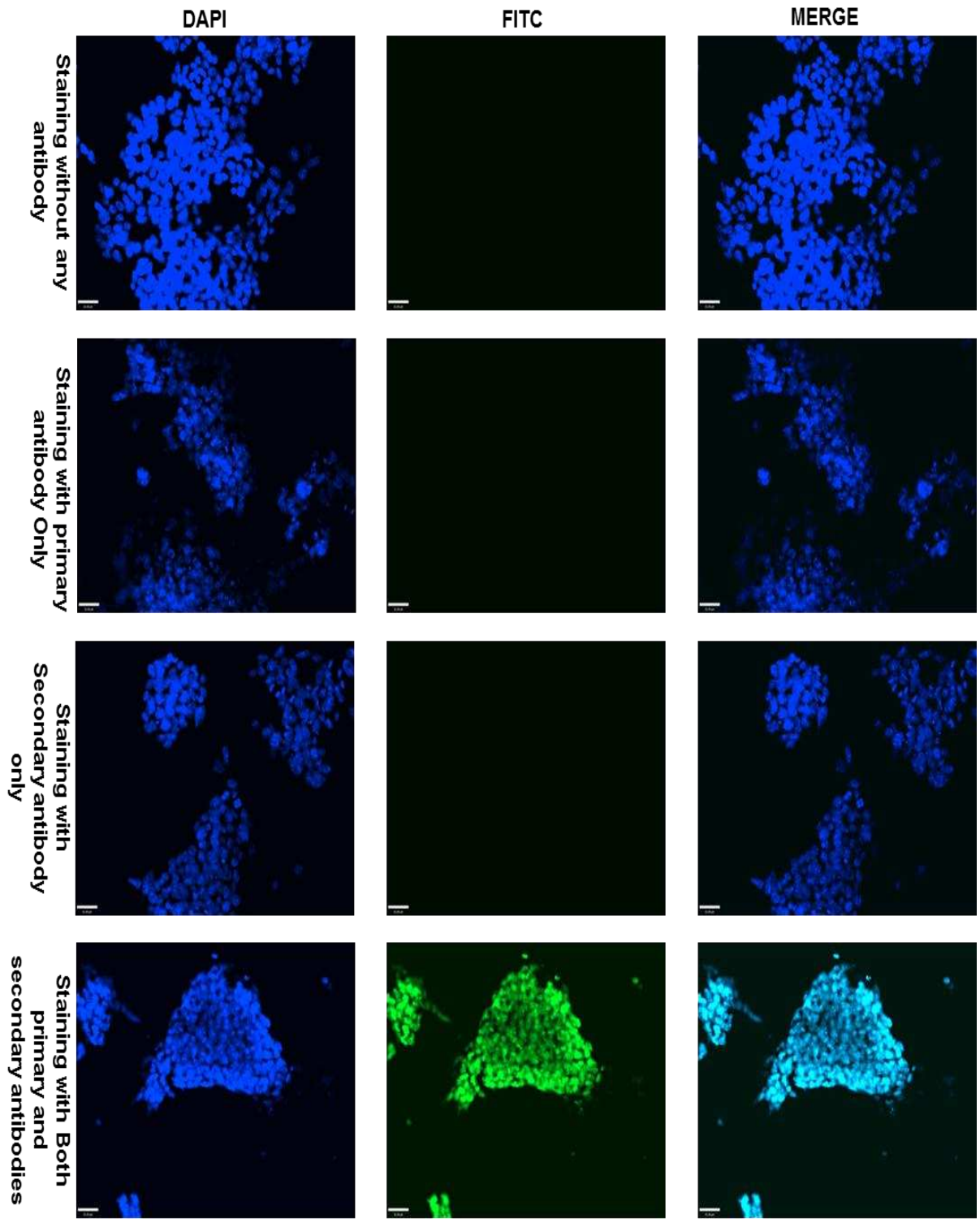


Figure 3.11: Mouse ES (E14tg2a) cells were used as positive control for Sox2 expression. ES cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). Only ES cells stained with both primary and secondary antibodies showed positive signal (All pictures were taken at 20X magnification and scale bar is 32µm).

3.3.3.2.Sox2 Negative

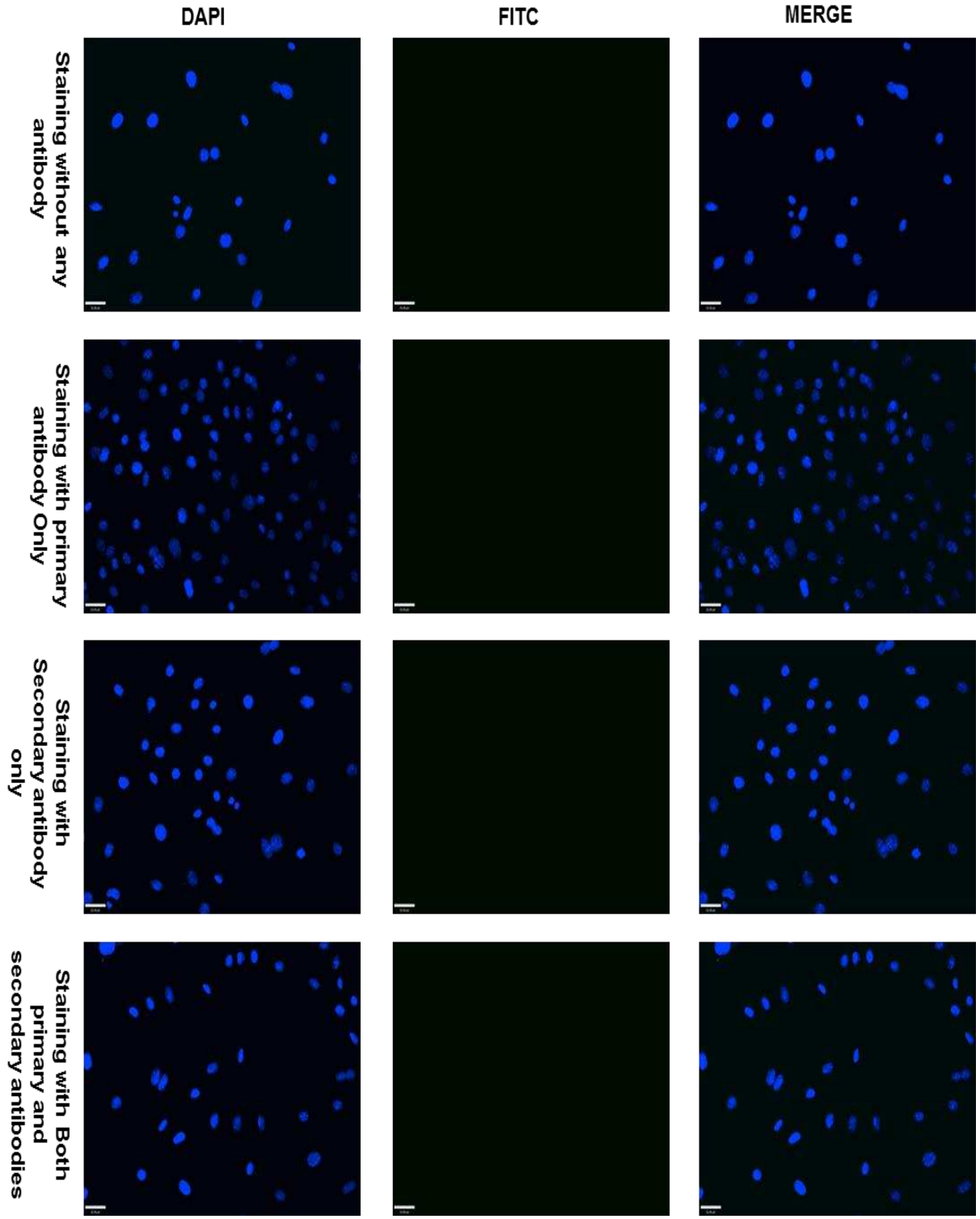


Figure 3.12: Primary mouse calvaria cells (kindly given by a colleague Omar Qutachi from Dr Lee Buttery research group) were used as negative control for Sox2 expression. Calvaria cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). No signal was detected (All pictures were taken at 20X magnification and scale bar is 32µm).

3.3.3.3.Sox9 Positive

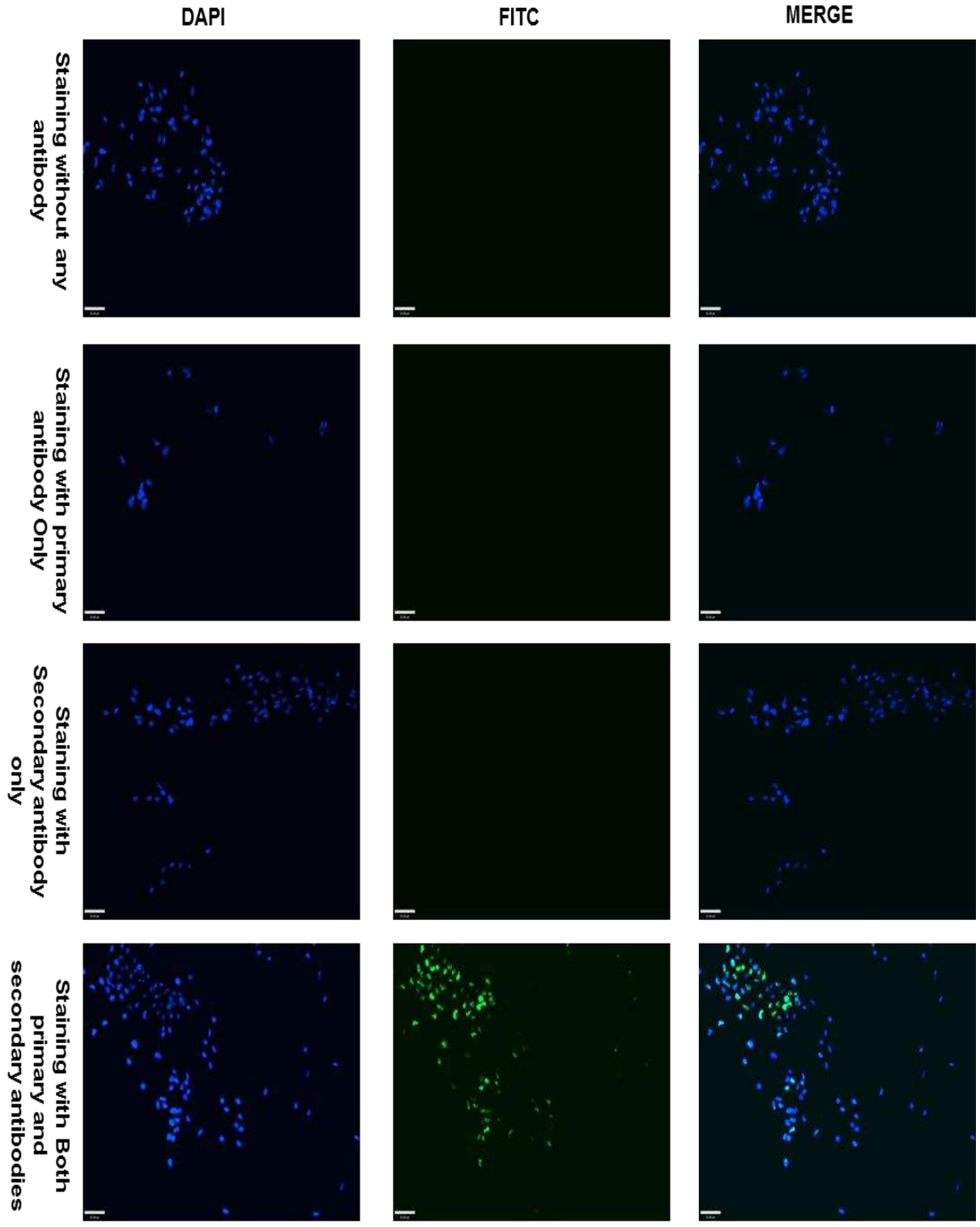


Figure 3.13: Mouse NSC cells isolated from lateral ventricle (kindly given by fellow research student Stephanie Strouhbecker from Dr Virginie Sottile research group) were used as positive control for Sox9 expression. NSCs were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox9). Only NSCs stained with both primary and secondary antibodies showed positive signal (All pictures were taken at 20X magnification and scale bar is 32µm).

3.3.3.4.Sox9 Negative

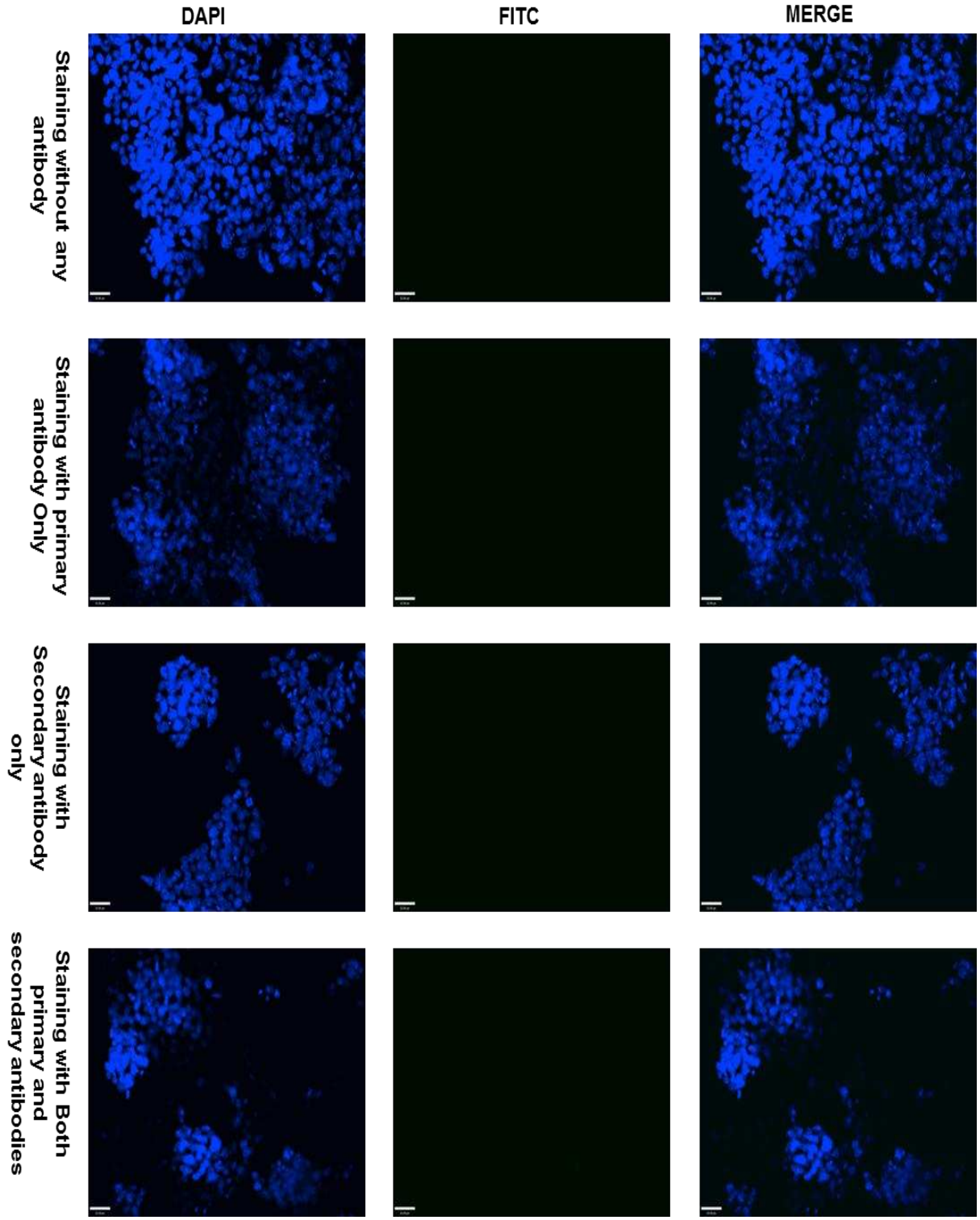


Figure 3.14: Mouse ES (E14tg2a) cells were used as negative control for Sox9 expression. ES were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox9). No positive signal was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

3.3.3.5.Osterix Positive

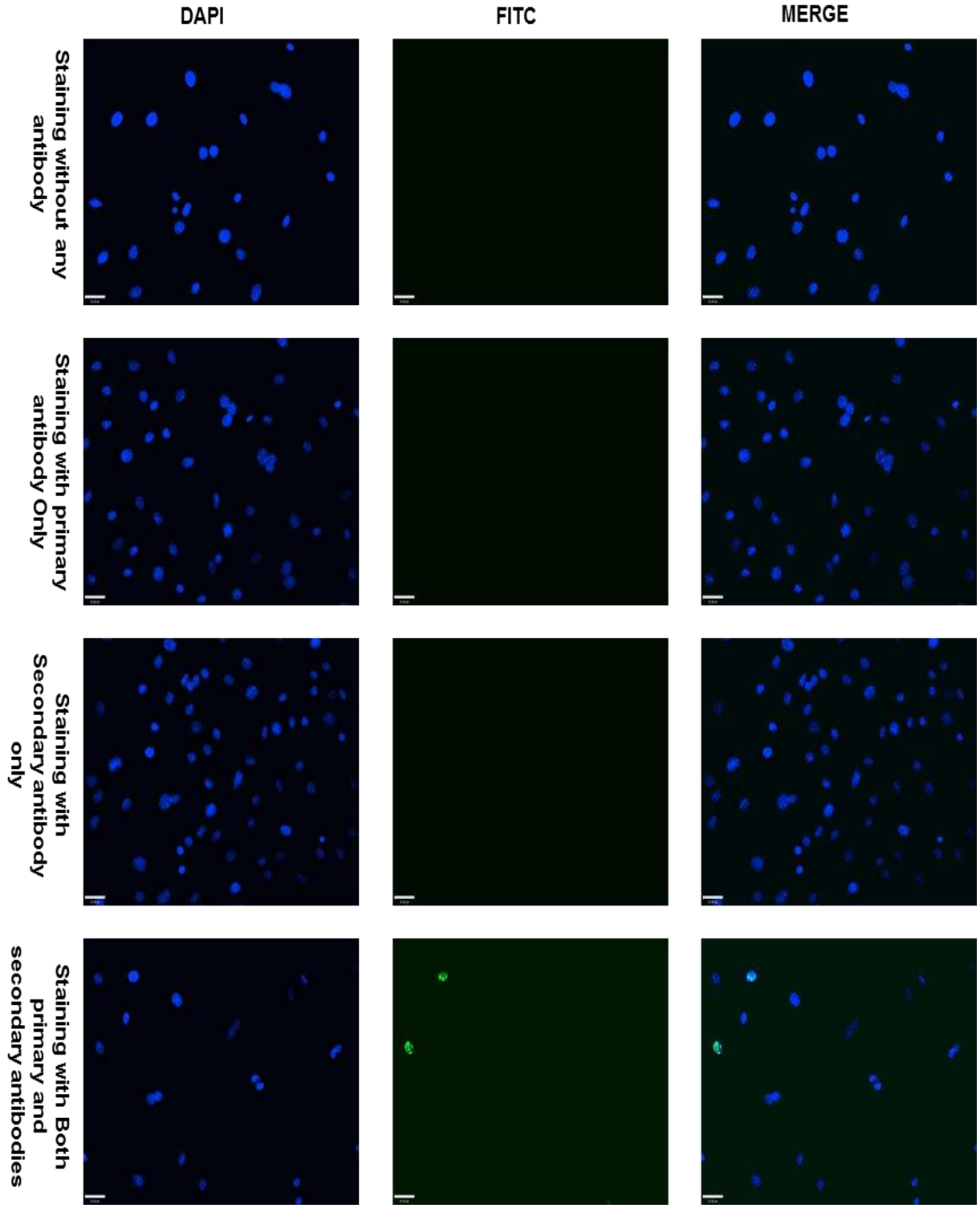


Figure 3.15: Mouse calvaria cells were used as a positive control for Osterix expression. Calvaria cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). Only few cells were found to be positively stained (All pictures were taken at 20X magnification and scale bar is 32µm).

3.3.3.6.Osterix Negative

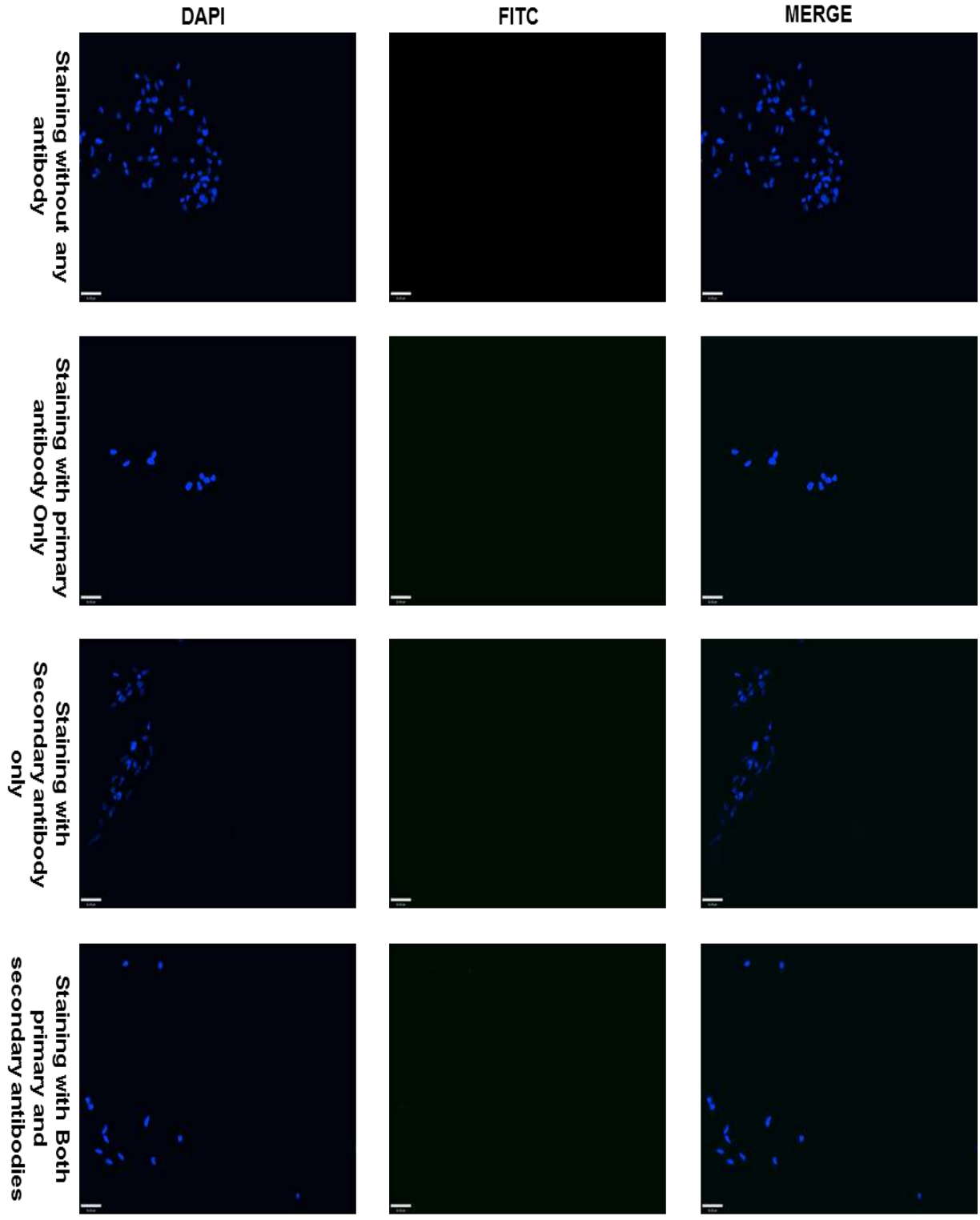


Figure 3.16: Mouse NSC (from lateral ventricle) cells were used as negative control for Osterix expression. NSCs were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox9). No signal was detected in any of the stained cells (All pictures were taken at 20X magnification and scale bar is 32µm).

3.3.3.7.Osteocalcin Positive

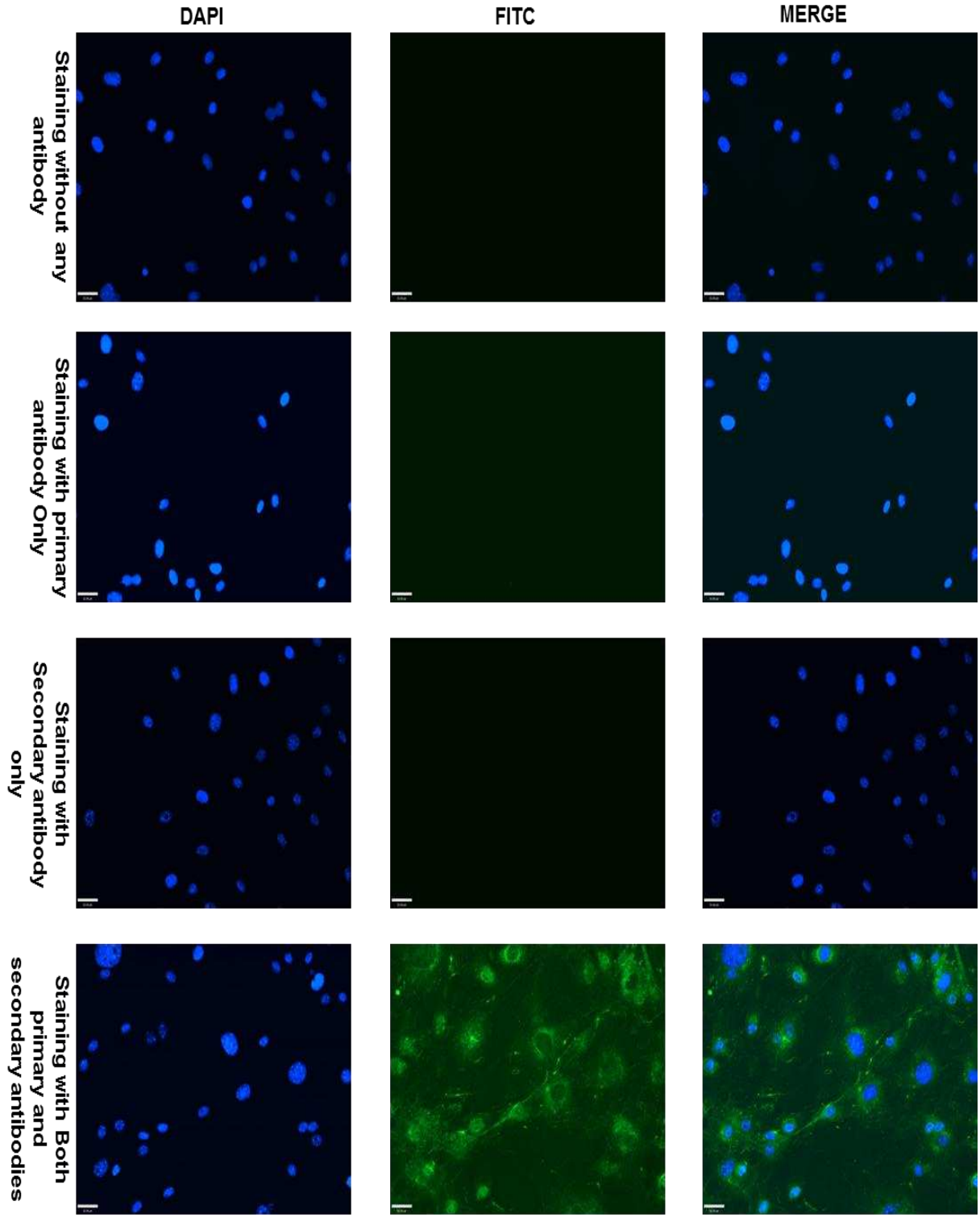


Figure 3.17: Primary mouse osteoblast cells (kindly given by a colleague Omar Qutachi) were used as a positive control for Osteocalcin expression. Osteoblast cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). Cells showed a cytosolic expression without any background (All pictures were taken at 20X magnification and scale bar is 32µm).

3.3.3.8.Osteocalcin Negative

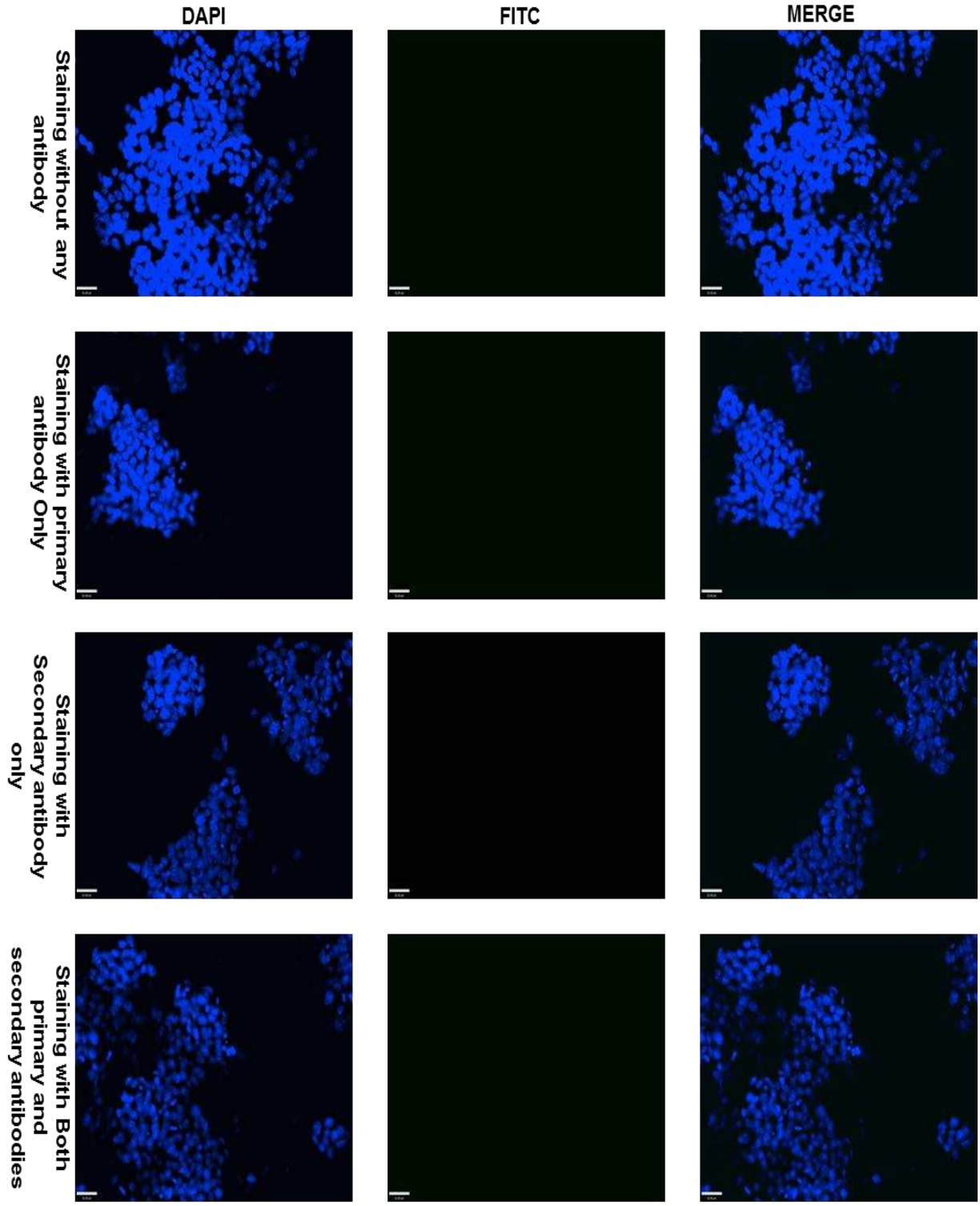


Figure 3.18: Mouse ES (E14tg2a) cells were used as negative control for Osteocalcin expression. ES were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Osteocalcin). No positive signal was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

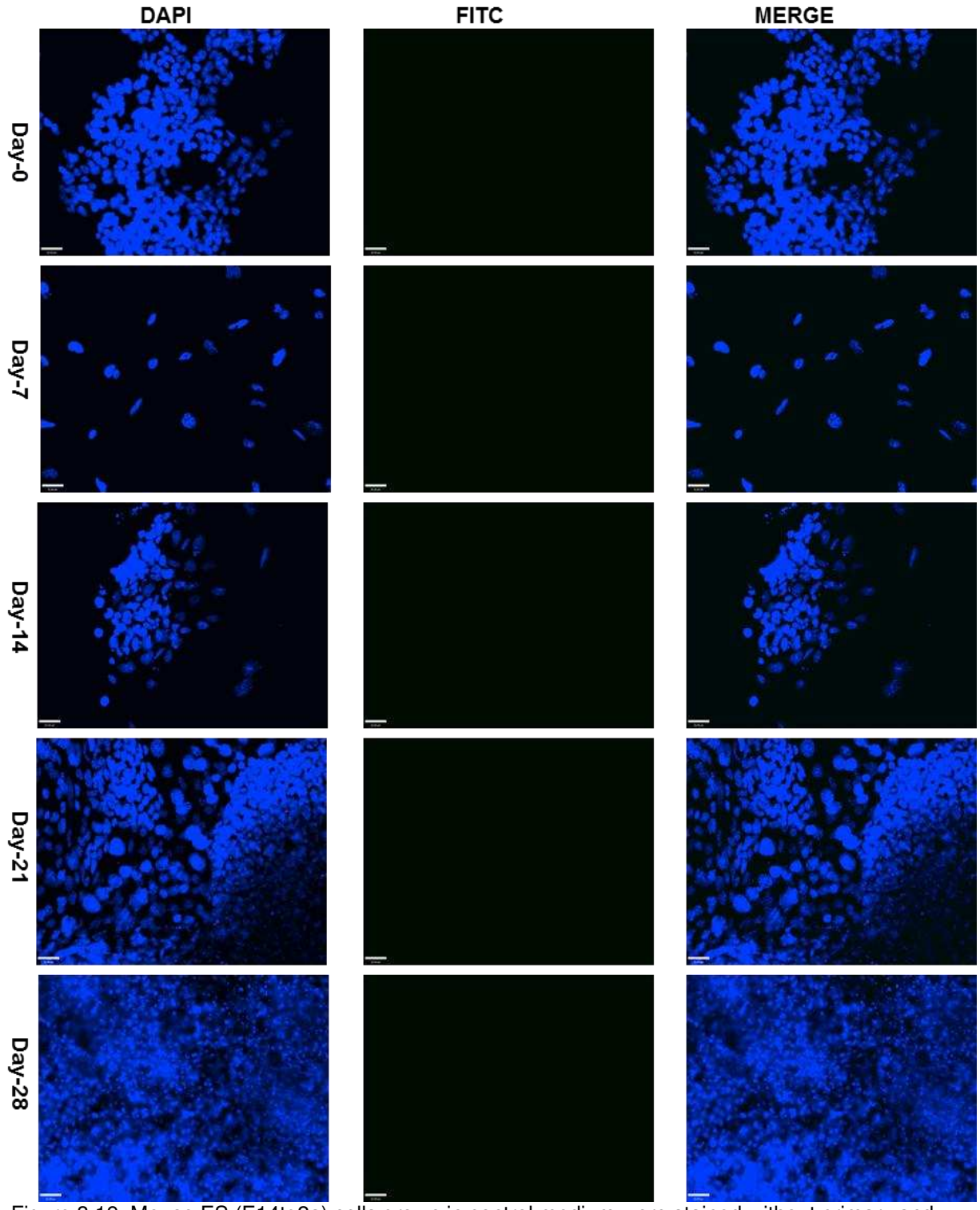


Figure 3.19: Mouse ES (E14tg2a) cells grown in control medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

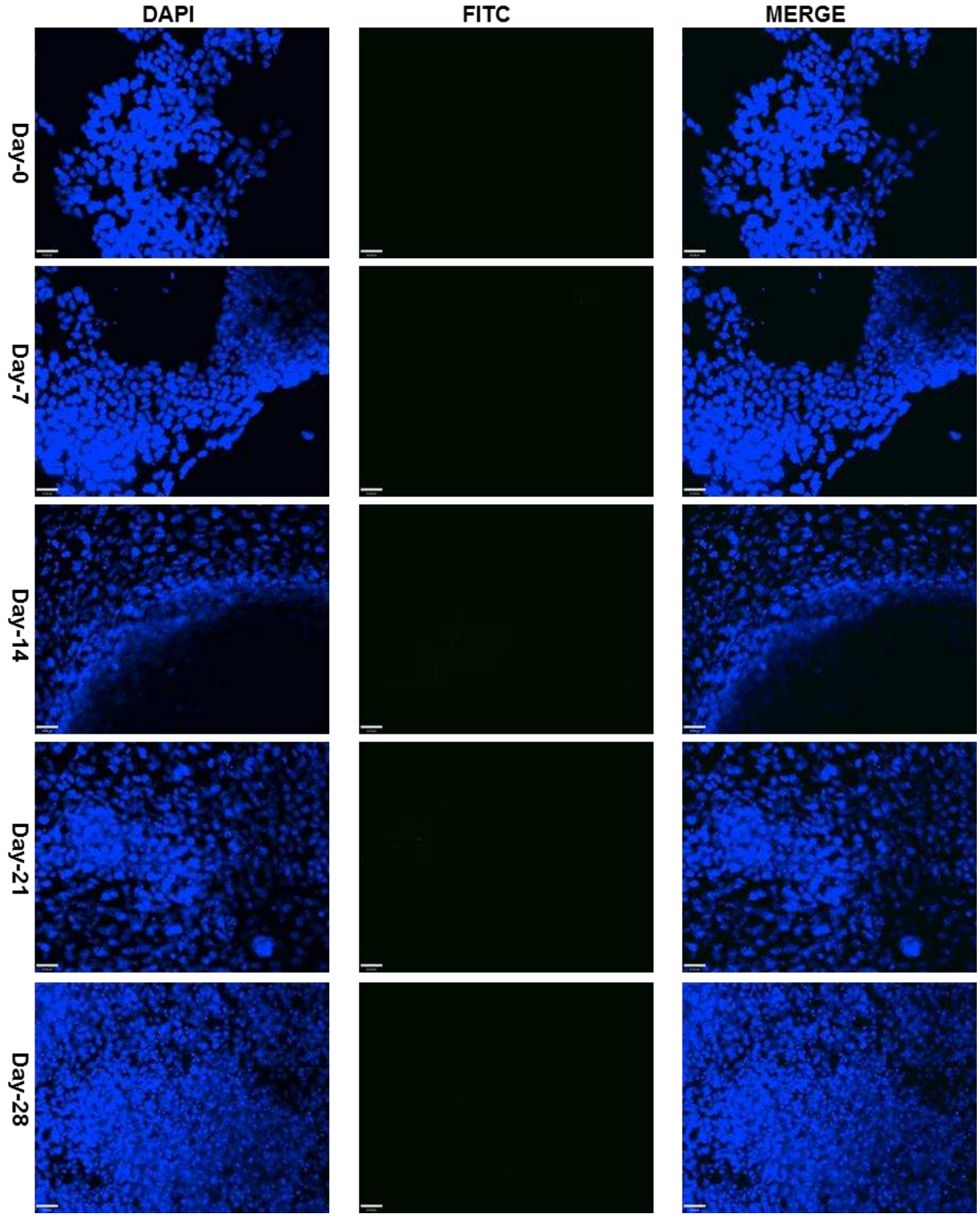


Figure 3.20: Mouse ES (E14tg2a) cells grown in OS medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

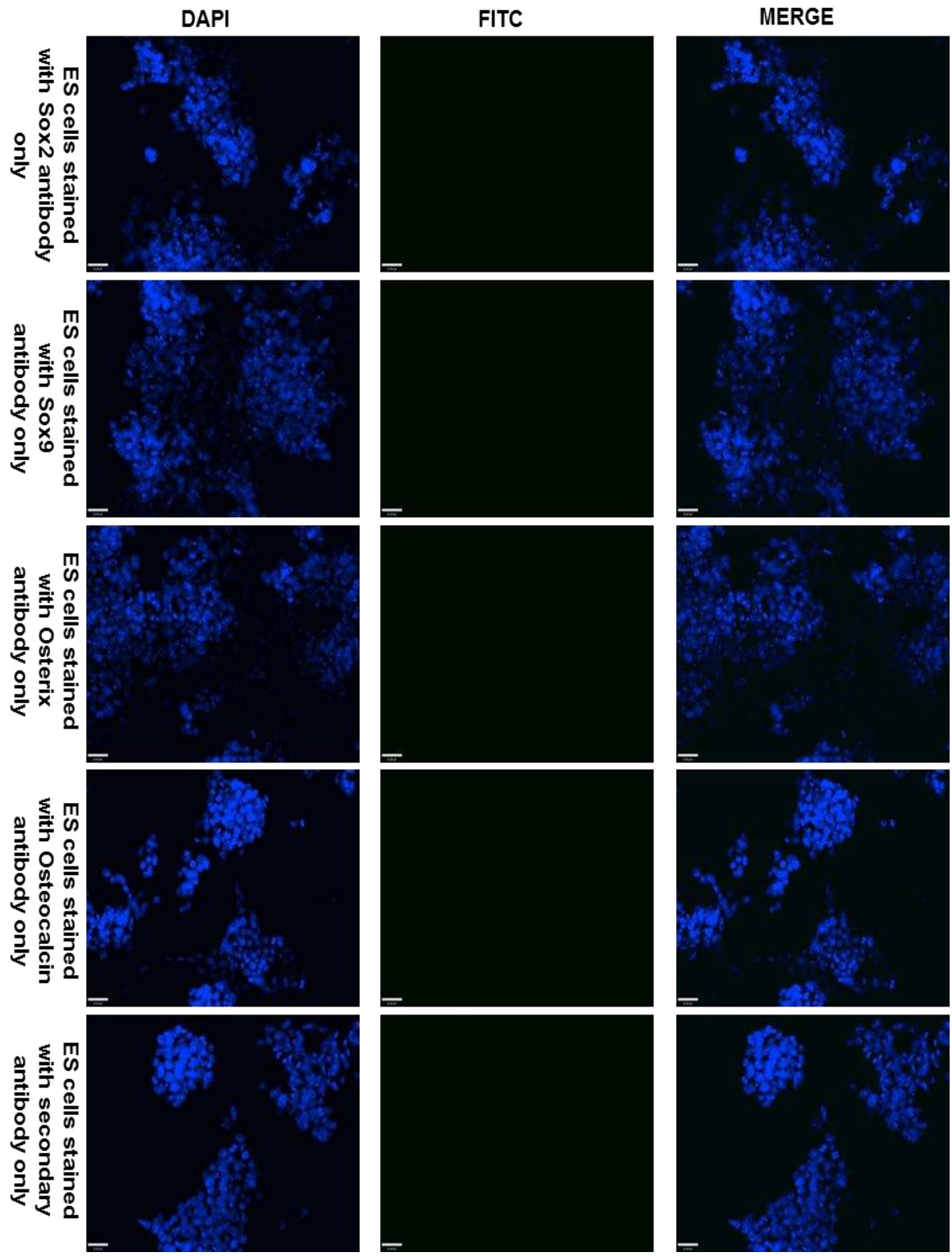


Figure 3.21: Mouse ES (E14tg2a) cells were stained with primary antibodies only and secondary antibody only to account for nonspecific fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

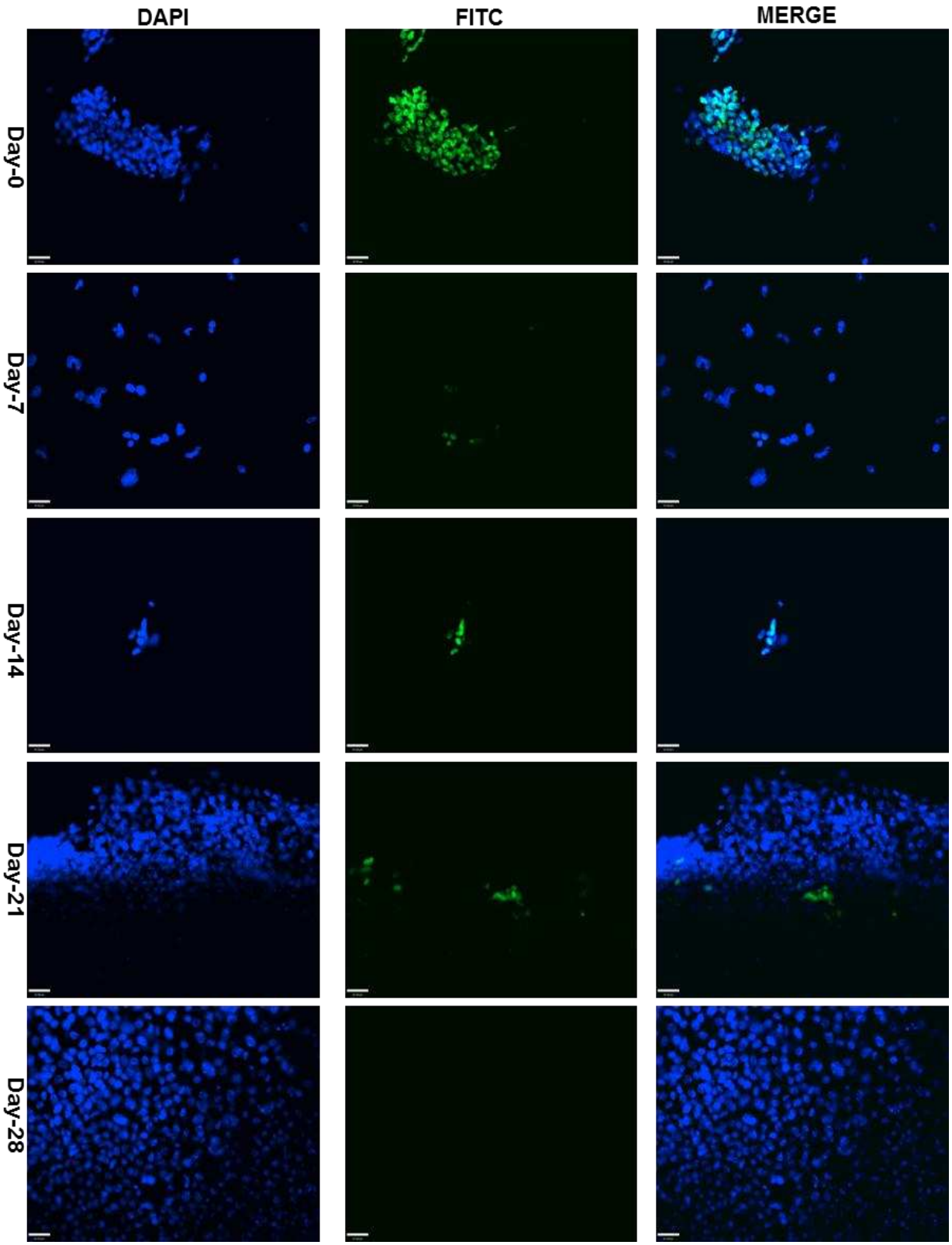


Figure 3.22: ESCs grown in medium lacking oesteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2) and found to be expressive at day-0 with a decreased level at day-7, day-14 and day-21 in cells. No signal for Sox2 was observed at day-28 (All pictures were taken at 20X magnification).

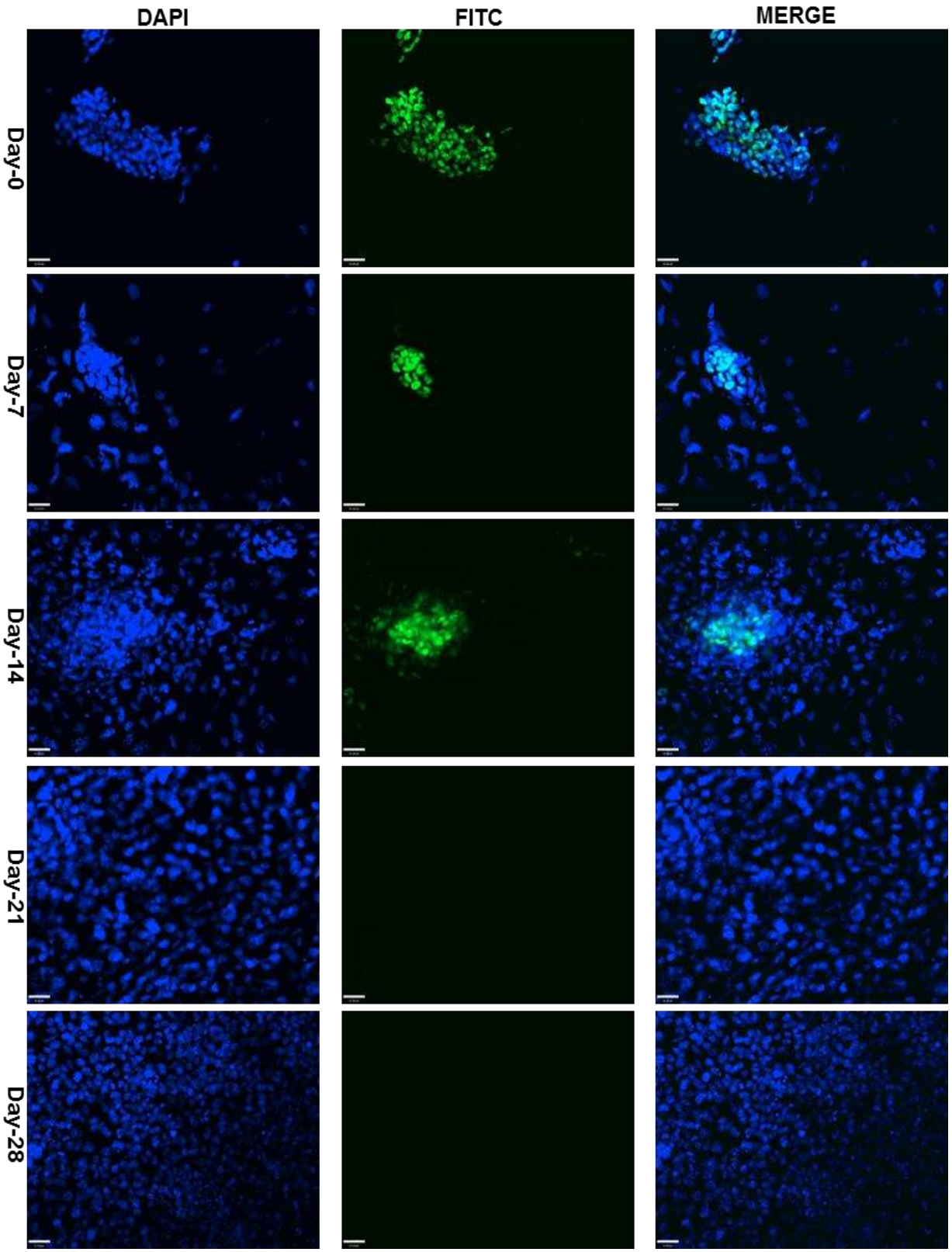


Figure 3.23: ESCs grown in medium containing oesteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2) and found to be expressive at day-0 with a decreased level at day-7 and day-14 in cells. No signal for Sox2 was observed at day-21 and day-28 (All pictures were taken at 20X magnification).

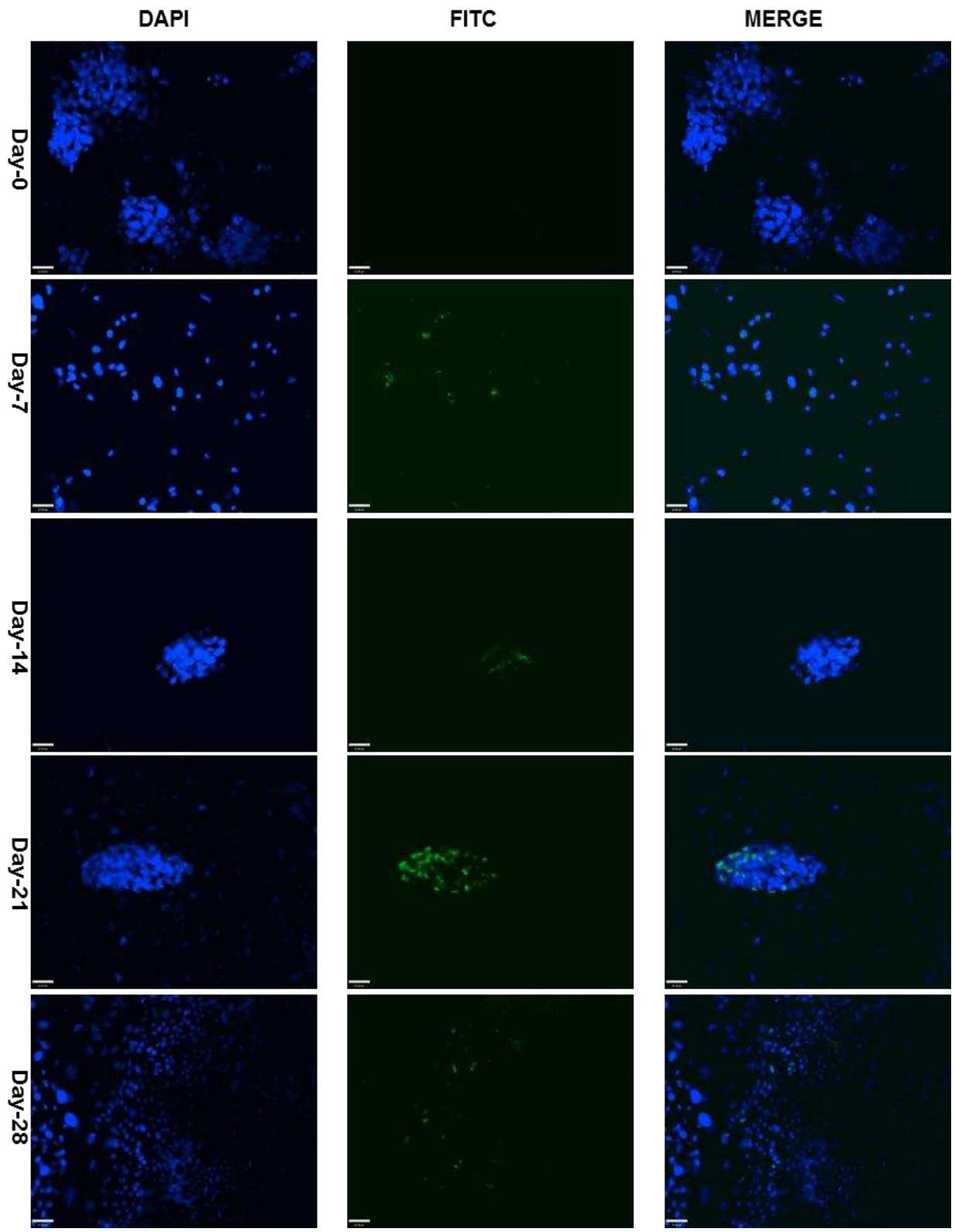


Figure 3.24: ESCs grown in medium lacking oesteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Expression of Sox9 was observed at all time points except at day-0 (All pictures were taken at 20X magnification). More cells appear to express Sox9 at day-21 than day-28.

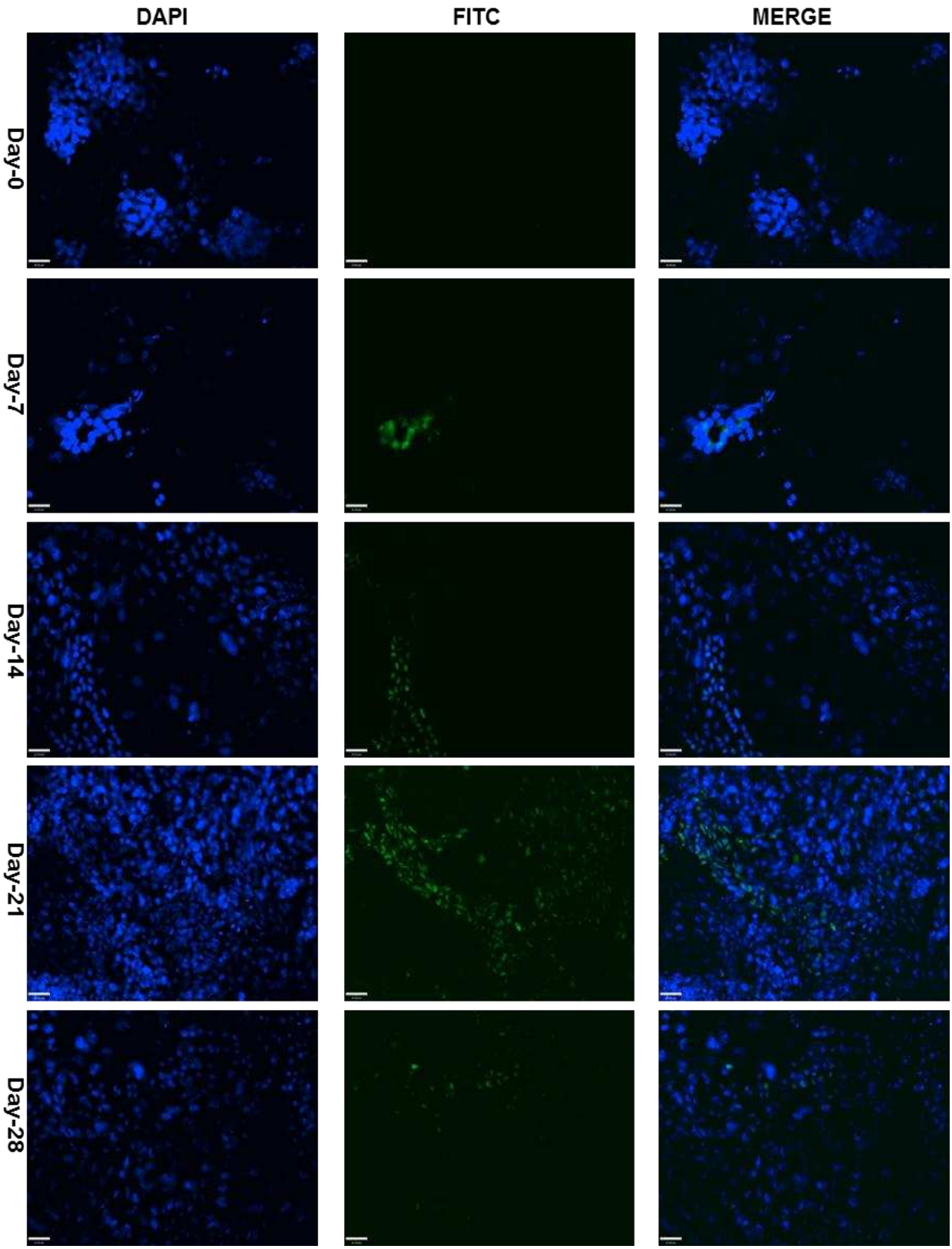


Figure 3.25: ESCs grown in medium containing oesteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Exprersion of Sox9 was observed at all time points except at day-0 (All pictures were taken at 20X magnification). It appears that number of Sox9 positive cells increased up to day-21 and then decreased at day-28.

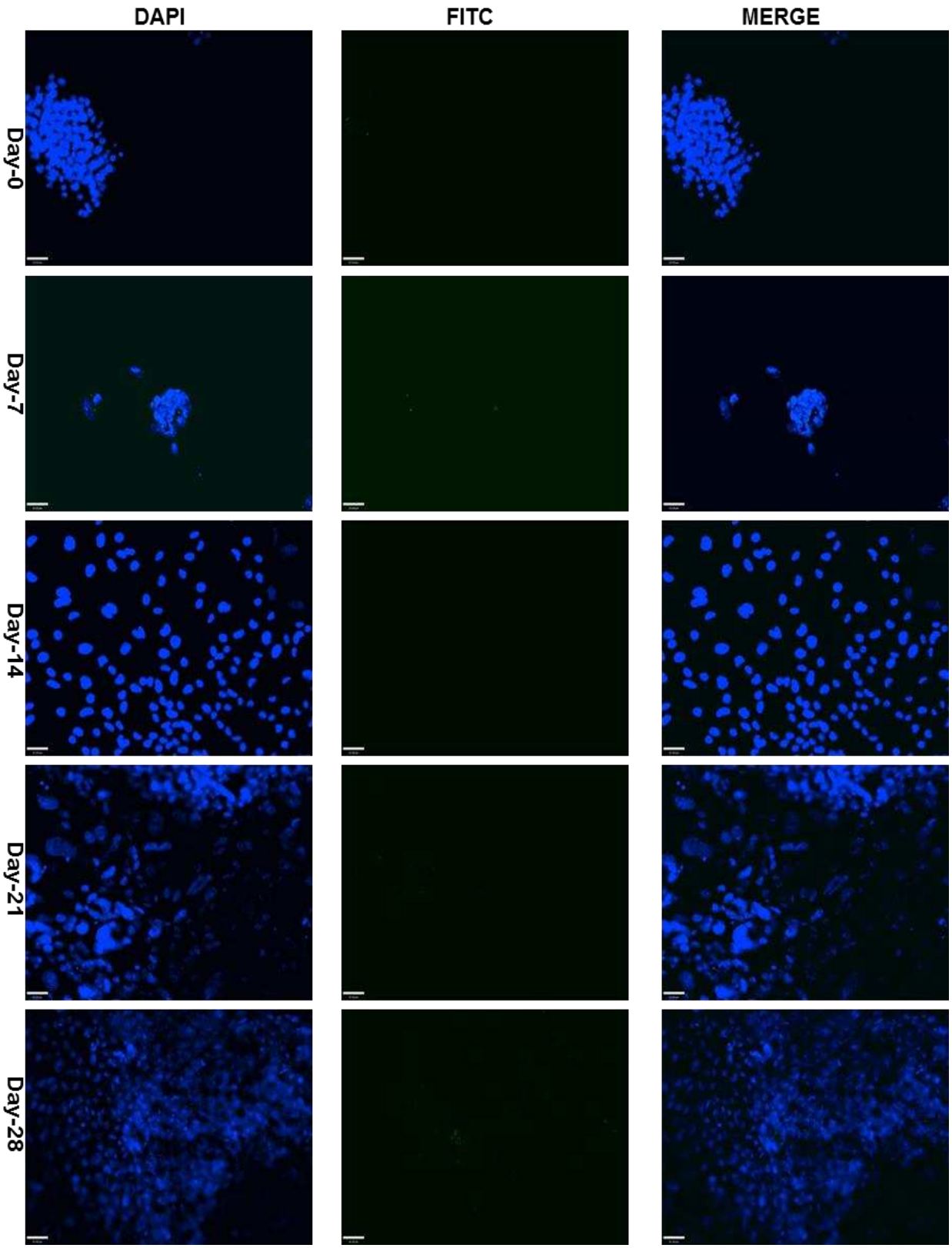


Figure 3.26: ESCs grown in medium lacking oesteogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No expression was observed at any time points selected for analysis (All pictures were taken at 20X magnification).

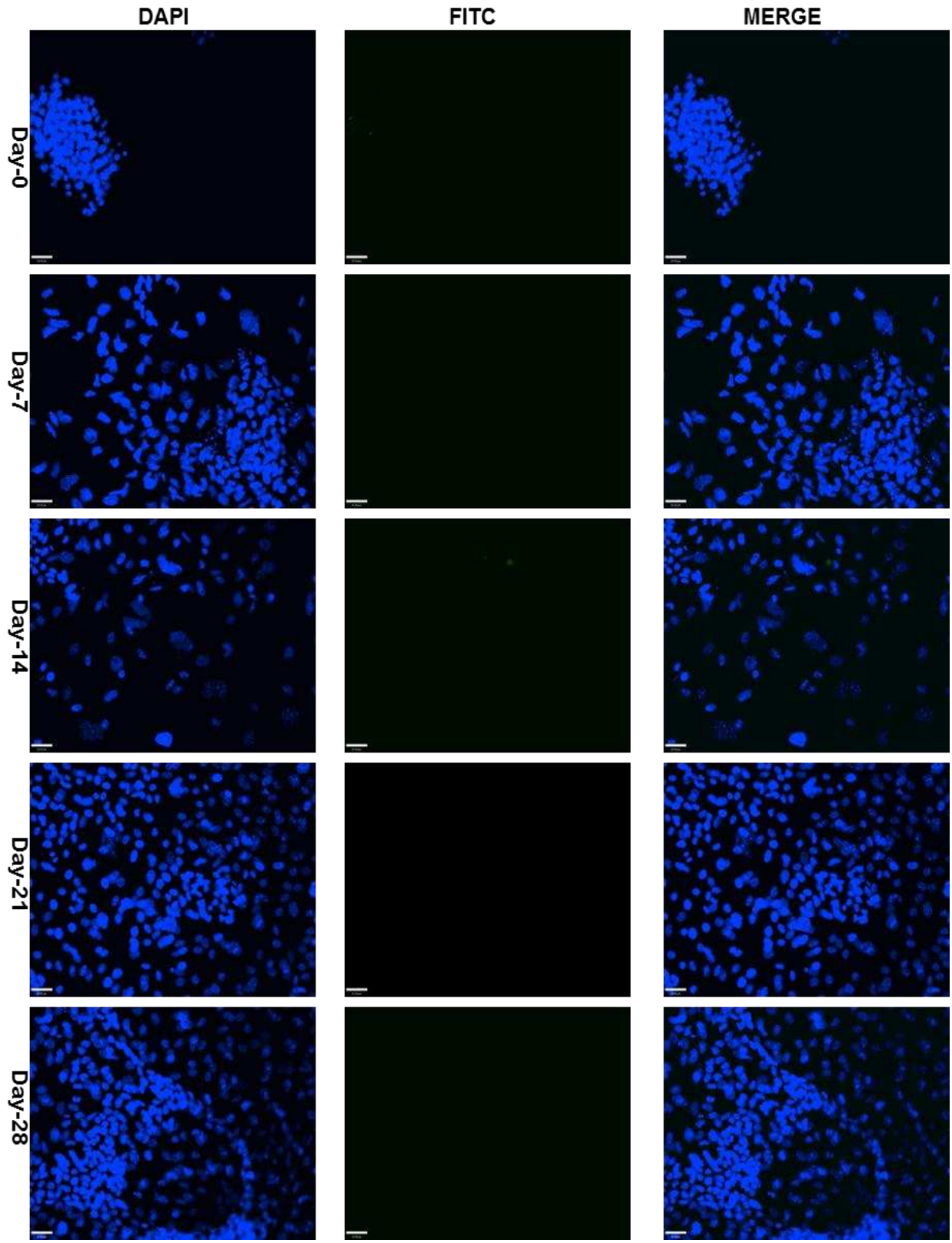


Figure 3.27: ESCs grown in medium containing oesteogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No expression was observed at any time points selected for analysis (All pictures were taken at 20X magnification).

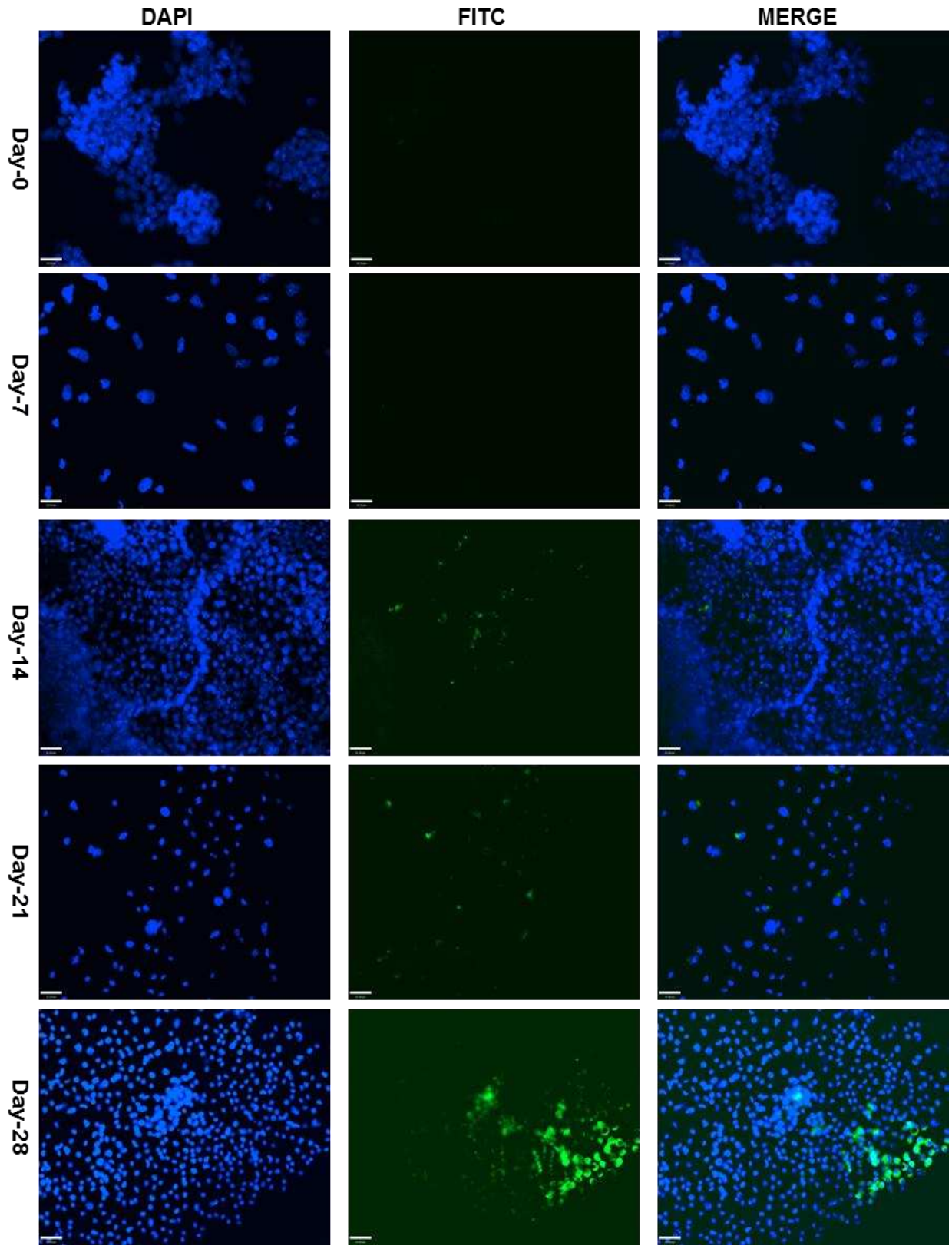


Figure 3.28: ESCs grown in medium lacking oesteogenic supplements stained using antibody against Osteocalcin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). With the exception of day-0 and day-7, all time points showed cytosolic expression which increased with time (All pictures were taken at 20X magnification).

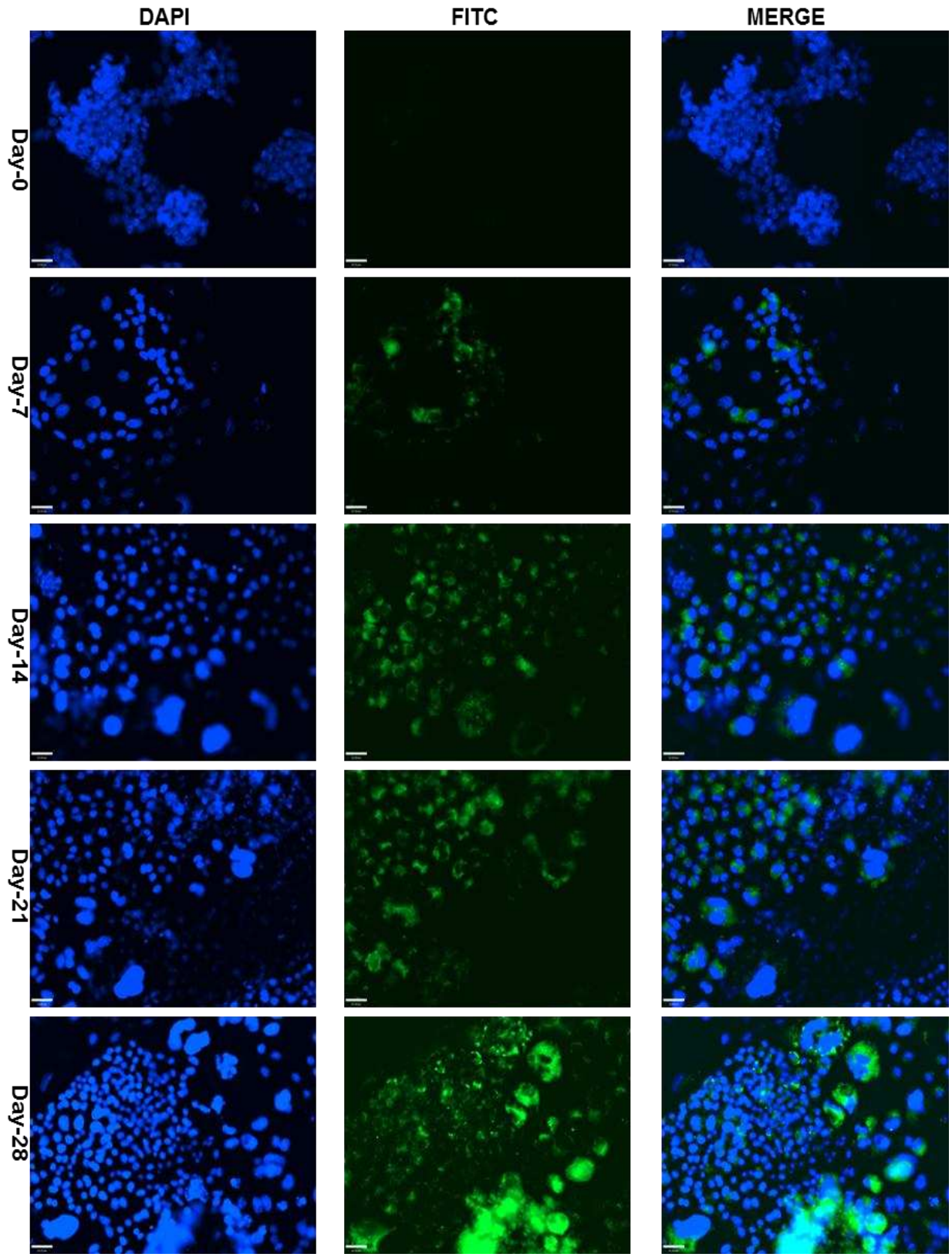


Figure 3.29: ESCs grown in medium containing oesteogenic supplements stained using antibody against Osteocalcin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). With the exception of day-0 all time points showed cytosolic expression of osteocalcin which increased with time (All pictures were taken at 20X magnification).

3.3.4. Molecular Analysis

3.3.4.1. Gene Expression Analysis by reverse transcription PCR (RT-PCR)

Gene expression studies of Oct4, Nanog and Sox2 as marker of pluripotency and Sox9, Alkaline phosphatase, Runx2, Osterix, Osteopontin and Osteocalcin as osteo-specific markers were carried out on random primed cDNAs. cDNA was prepared using RNA extracted from ES cells undergoing differentiation at different time points as shown in Figure 3.22. A house keeping gene GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was used as endogenous control. GAPDH was selected as invariable control because this differentiation protocol is based on previous studies which had also employed the same for normalizing gene expression analysis data (Buttery et al., 2001; Bourne et al., 2004). In addition other separate studies carried out to specifically compare different housekeeping genes in differentiating mouse embryonic stem cells to select for best reference gene have also shown that GAPDH is more reliable choice as normalization control over other reference genes such as HPRT and beta tubulin (Murphy et al., 2002; Willems et al., 2006). It appears from Figure 3.30 that GAPDH expression levels did not remain same throughout the differentiation time course which could be more likely due to amount of input used for PCR reaction. All gene products after PCR reaction were loaded on to ethidium bromide containing agarose gels and intensity of DNA bands were then calculated using AIDA, an image analysis software (raytest, Germany). Semi-quantitative gene expression analysis was carried out by normalizing the DNA band intensities values to GAPDH and ratio of product intensities plotted against days of treatment to obtain an expression profile in differentiating cultures and shown in Figures 3.31-3.39.

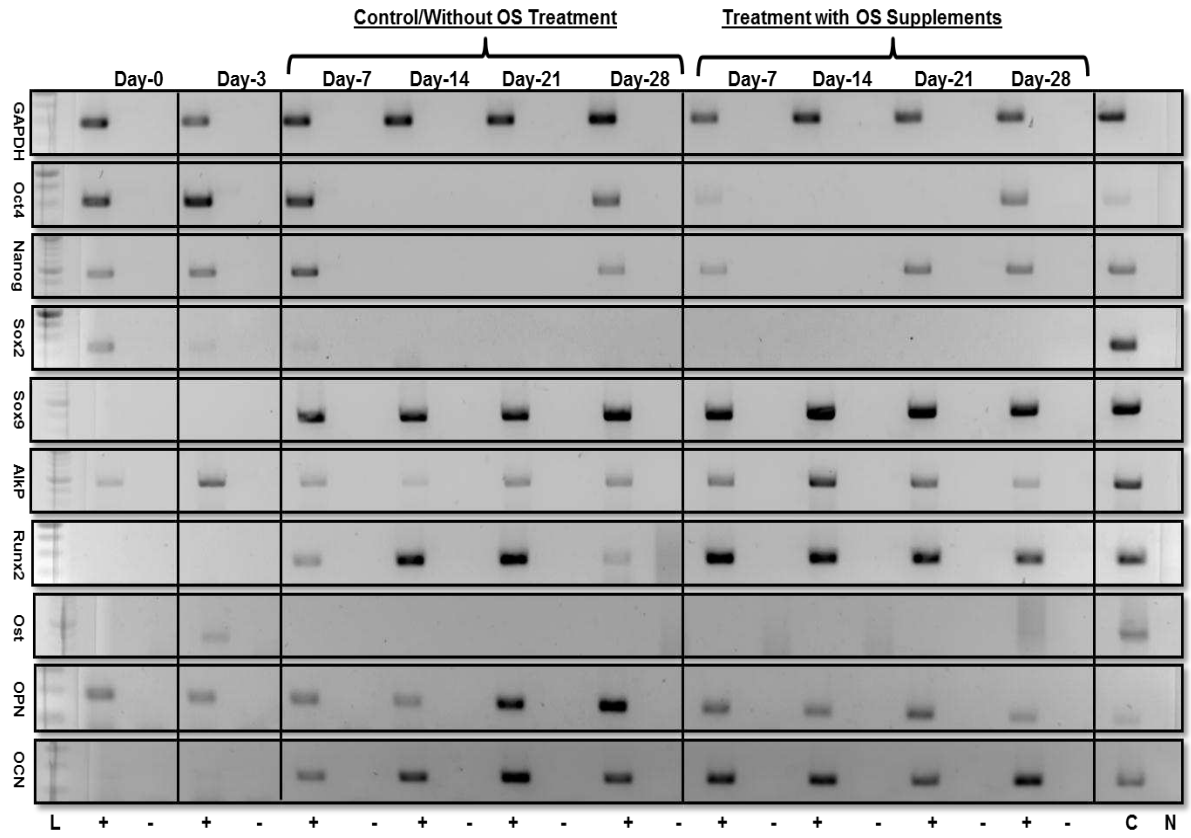


Figure 3.30: Gene expression analysis using random primed cDNAs of ESC osteogenic time-course. This analysis was carried out in triplicates for three independent biological replicates (n=3) and semi-qunatitiative analysis is shown below (Figures 3.31-3.39). Note: + and – refers to RT and –RT respectively whereas C is Positive control, N is PCR Negative and L is 100bp DNA Ladder.

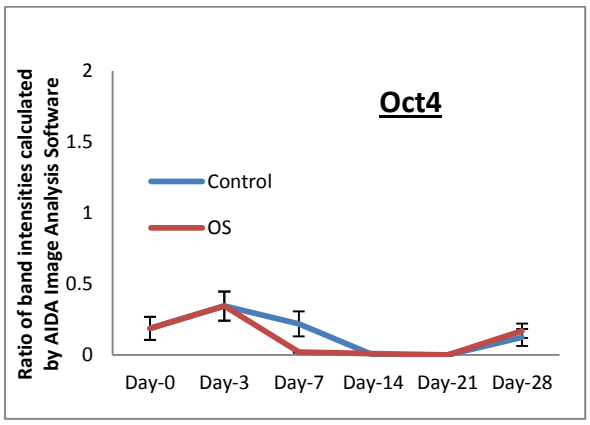


Figure 3.31: Semi-quantitative gene expression analysis showing expression of Oct4 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

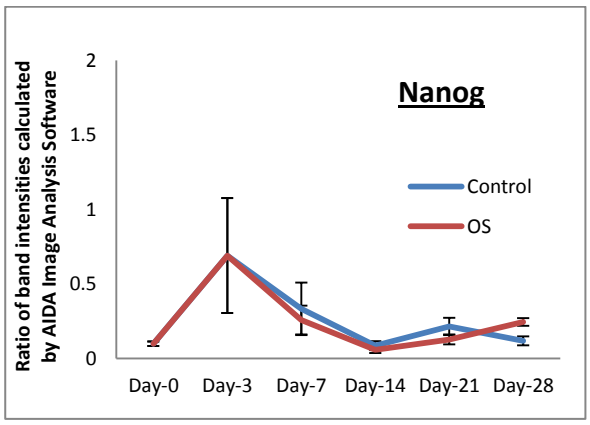


Figure 3.32: Semi-quantitative gene expression analysis showing expression of Nanog in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

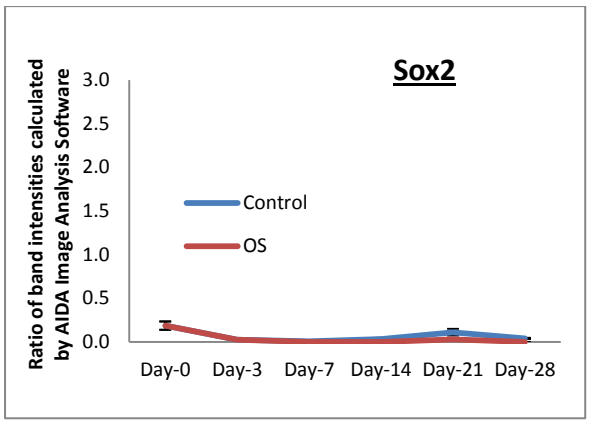


Figure 3.33: Semi-quantitative gene expression analysis showing expression of Sox2 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

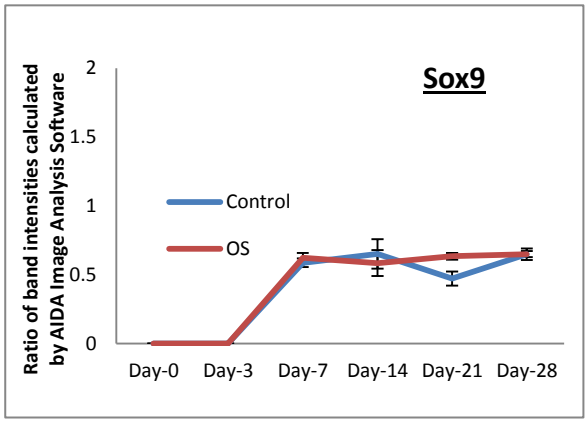


Figure 3.34: Semi-quantitative gene expression analysis showing expression of Sox9 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

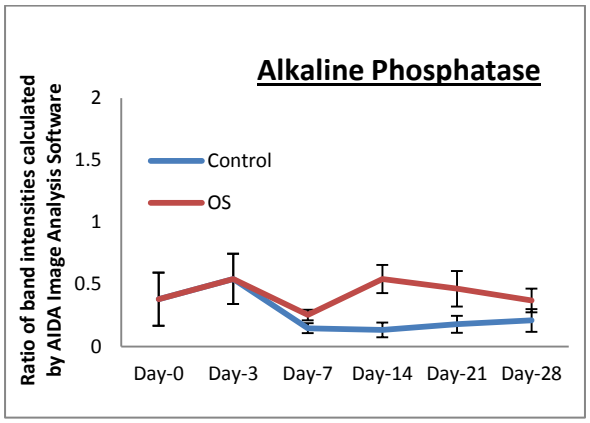


Figure 3.35: Semi-quantitative gene expression analysis showing expression of Alkaline Phosphatse in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

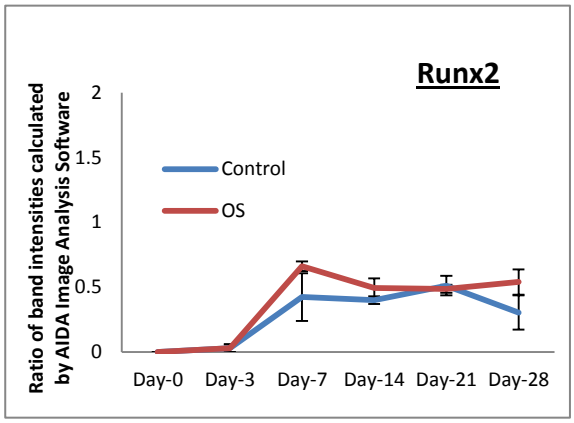


Figure 3.36: Semi-quantitative gene expression analysis showing expression of Runx2 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

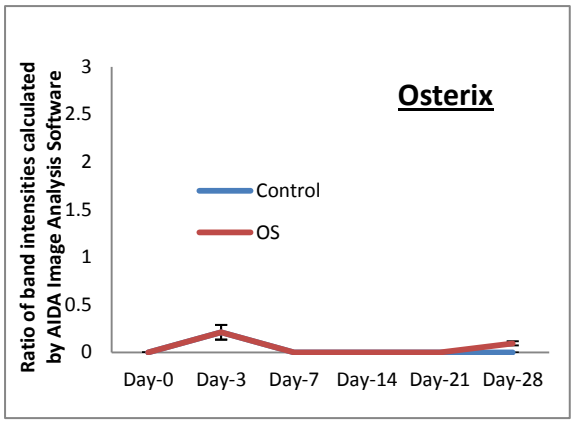


Figure 3.37: Semi-quantitative gene expression analysis showing expression of Osterix in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

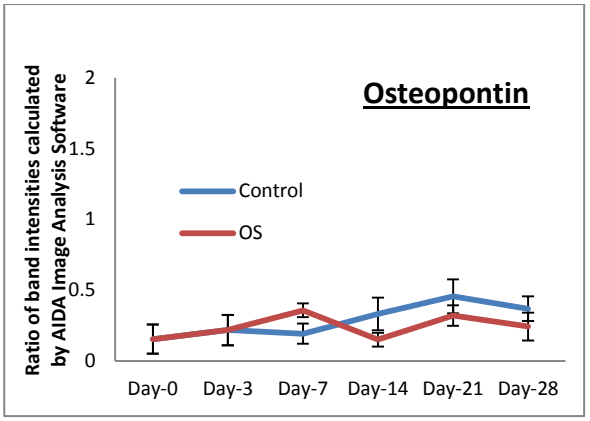


Figure 3.38: Semi-quantitative gene expression analysis showing expression of Osteopontin in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

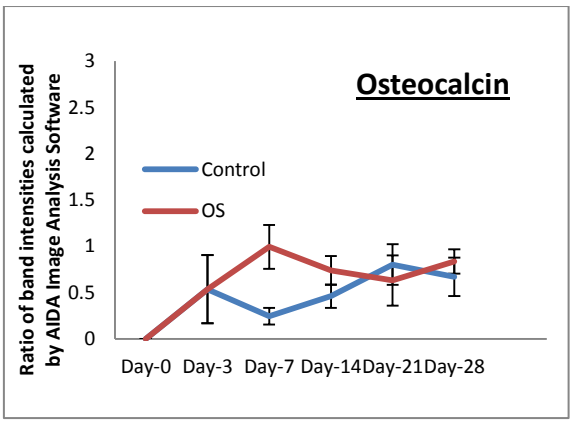


Figure 3.39: Semi-quantitative gene expression analysis showing expression of Osteocalcin in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean±SEM.

Oct4 was found to be strongly expressed in undifferentiated cells and then subsequently becomes down regulated as cells differentiate and seemed to up regulate again after day-21. In both control and OS treated cells the pattern of RNA expression seemed to be the same with a sharp reduction in level of expression after day-7 (levels beyond the resolution of this assay at day-14 and day-21) but seemingly up regulated at day-28 (Figures 3.30 and 3.31).

Nanog displayed a pattern of expression similar to Oct 4 in control cells, with high detectable levels in day3 and day7, no expression in day14 and day 21 cells and expression back on at day 28. Similarly, in OS-treated cells expression of Nanog is lost after day 7 as observed in control cells, but resumed at day21 and maintained in day28 cells (Figures 3.30 and 3.32).

Sox2 expression was detected in undifferentiated E14Tg2A (day-0) and found to be down regulated in both control and OS cells after day-3 (Figures 3.30 and 3.33). Though Sox2 protein was found to be present in cells grown in control medium at day-7, day-14 and day-21 with fewer cells being positive past day-0 (Figure 3.22). Sox2 protein was not detected after day-14 in OS-treated population (Figure 3.23), and this correlates with lack of RNA detection at these time points by the RT-PCR (see discussion section 3.4 for further details).

Sox9 expression at RNA level was observed by RT-PCR throughout the differentiation time course in E14Tg2A grown in medium with and without OS supplements (Figures 3.30 and 3.34) except day-0 and day-3. These results are in agreement with those obtained by immunofluorescence where Sox9 protein was also found to be present at all time-points starting from day-7 to day-28 in both treated and untreated cells (Figures 3.24 and 3.25).

Alkaline phosphatase was found to be present at all time-point examined with significant up regulation in second week of differentiation in OS treated cells as shown in Figures 3.30 and 3.35.

Runx2 expression was undetectable at day 0 and day 3 of differentiation and was found to be peaking up after two weeks of differentiation in both control and OS treated cell populations and then it appeared to be down regulated after day-21 (Figures 3.30 and 3.36).

Osterix expression was not detected at earlier time points in E14Tg2A growing in both kinds of medium, containing and lacking OS supplements as shown in Figures 3.30 and 3.37 though a faint signal was observed at day-3 and very little at day-28 in OS cells. As previously described, osterix protein was not found to be present at any time point during differentiation of ES cells (Figure 3.26 and 3.27).

Osteopontin was found to be present in undifferentiated as well as differentiated cells. There appeared to be a marked increase in expression at day-21 and day-28 in control cells whereas observed to be down regulating in OS cells past day-21 as is shown in Figures 3.30 and 3.38.

Osteocalcin expression was first observed at day-7 in OS differentiated cells and then found at all time-points (Figures 3.30 and 3.39). This correlates well with osteoclacin protein expression detected by immunofluorescence as shown earlier in Figure 3.28 and 3.29. Protein expression was found to be enhanced at late stages of differentiation i.e. at day-21 and day-28 in both control and OS cells. OS-treated cells were found to be expressing more osteocalcin protein than non-treated cell populations.

3.3.4.2. Methylation sensitive PCR (MS-PCR) for methylation analysis

In order to determine whether DNA methylation could play a role in regulating expression of Sox2 as E14Tg2A cells grow in medium prompting oesteogenic differentiation, two regulatory regions of Sox2 (SRR1 and SRR2) were analysed by methylation sensitive PCR. For this first, genomic DNA is digested with MspI and HpaII restriction enzymes which are

isoschizomers but MspI activity is not affected by DNA methylation at its target site. HpaII is unable to cleave its target sequence when it is methylated. This difference can be exploited to investigate the methylation status of the sequence in question, as a fragment containing Msp sites should never be amplified following digestion with MspI, but can be amplified after HpaII digestions if all the sites are methylated. Three PCR reactions were run in parallel for these three set of samples using primers specific for either control sequences, SRR1 and SRR2. Uncut (U) refers to mock digested genomic DNA which has not received any enzymatic treatment, M referes to genomic DNA digested with MspI and H is for gencomic DNA digested with HpaII restriction enzyme (Figures 3.40-3.43).

To determine the quality of DNA, efficiency and success of restriction reaction, controls were included in PCR reaction that can selectively amplify the regions of digested DNA without any MspI/HpaII sites, sites that are always unmethylated and sites that are always methylated. For this purpose a region of DNA is selectively amplified using mAprt primers which have been reported to contain MspI/HpaII sites but remain unmethylated (Macleod et al., 1994). And a sequence of known methylation status namely insulin like growth factor receptor differentially methylated region 2 (Igf2R-DMR2) from mice was selected as a control since this region becomes methylated during oogenesis and remains methylated (Feil et al., 1994).

As can be seen in Figure 3.40 that region of mAprt known to be unmethylated did not amplify in both MspI and HpaII digested DNA samples at any time point in both OS-treated and not treated cell populations. This indicates that digestion is not partial and any product obtained with primers specific for Sox2 regulatory regions would be due to their methylation.

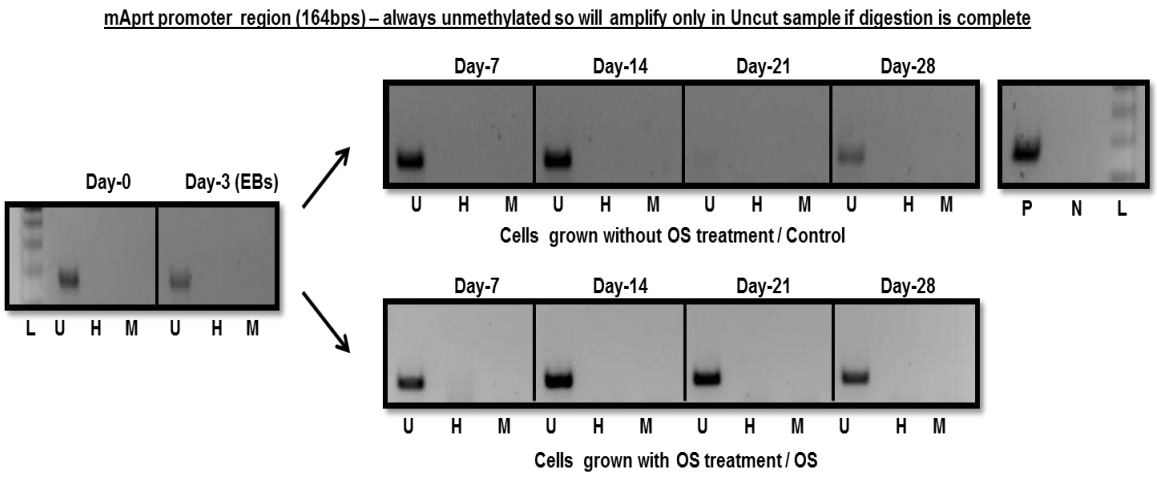


Figure 3.40: mAprt promoter region used as control and showing that no amplified product was observed in *Msp*I and *Hpa*II digested samples so digestion is complete.

Figure 3.41 shows the amplified product form mIgf2R region in HpaII digested samples and not in corresponding MspI digested samples. This also indicates that any product with primers specific to Sox2 regulatory regions in HpaII digested DNA samples would be due to methylation at Msp sites and not due to incomplete digestion.

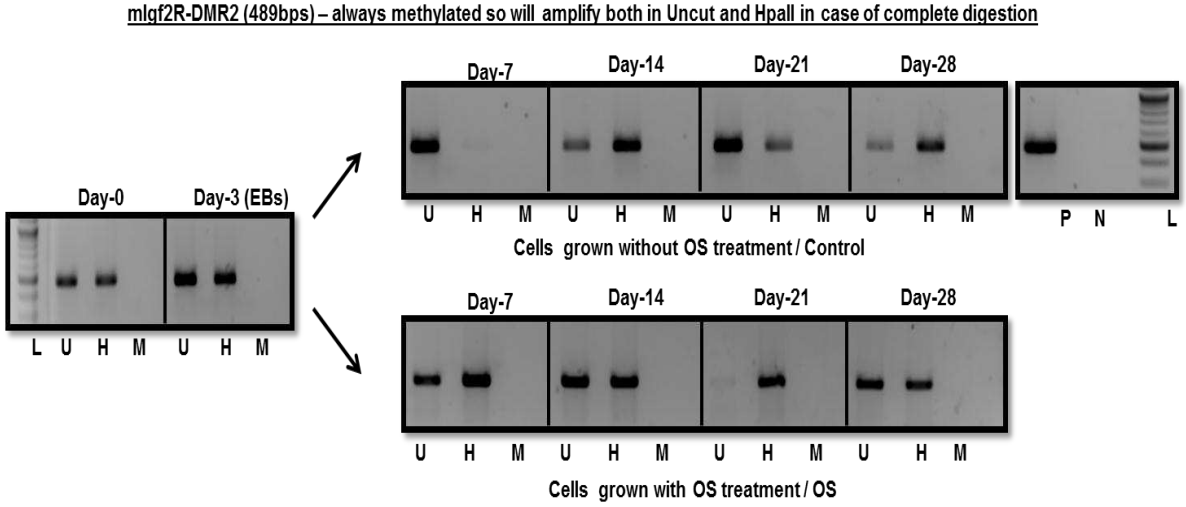


Figure 3.41: mIgf2R-DMR2 region used as control and showing that no amplified product was observed in *Msp*I digested samples so digestion is complete.

SRR1 and SRR2 as alreqady detailed in section 1.5.3 are two evolutionary conserved regualotry regions of Sox2. Both of these regions i.e. SRR1 and SRR2 were found to be

methylated at all time-points analysed in both non-treated and OS-treated cell populations except day-0 and day-3 as shown in Figure 3.42 and Figure 3.43.

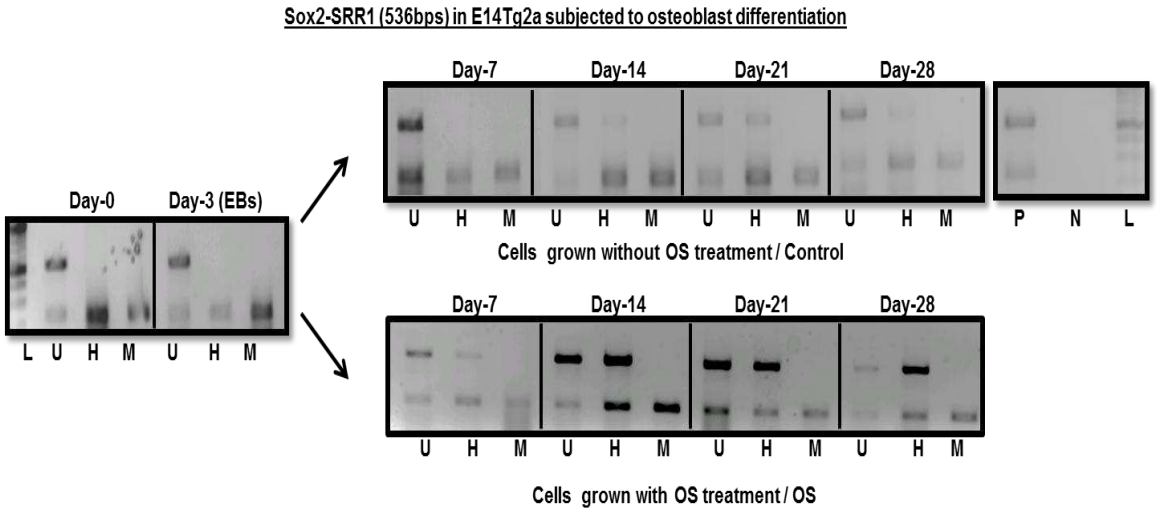


Figure 3.42: SRR1 region of Sox2 was found to be methylated at all time-points examined by MS-PCR (day-7 of control could be due to less amount of DNA input in PCR reaction)

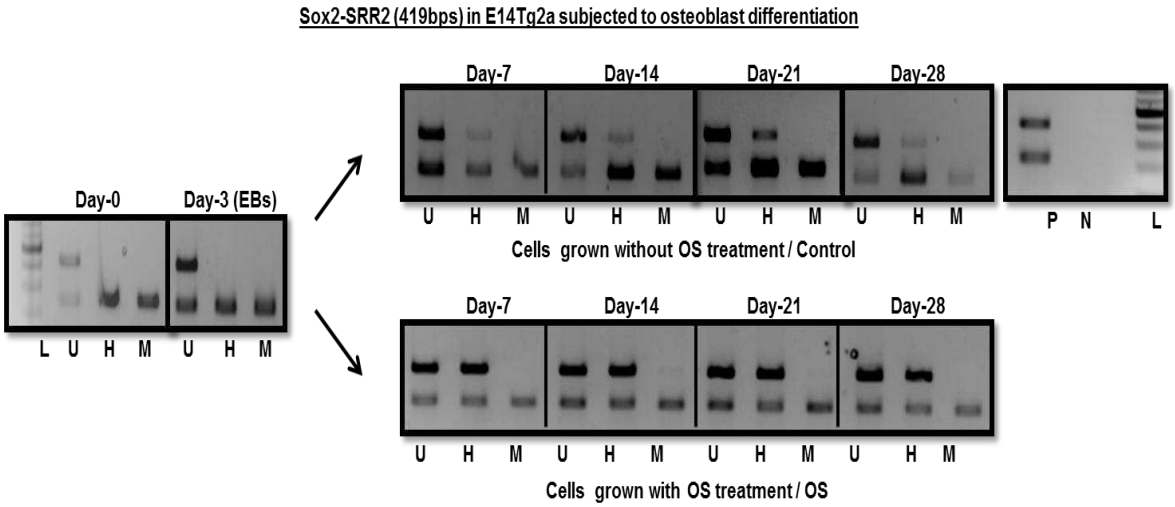


Figure 3.43: SRR2 region of Sox2 was found to be methylated at all time-points examined by MS-PCR (Key: U – Uncut Sample of DNA; M – *Msp*I digested DNA; H - *Hpa*II digested DNA; P – Positive Control; N – PCR Negative Control; L – 100bp DNA Ladder)

3.3.4.3. Bisulphite Sequencing of SRR2 region

In order to further confirm and analyse the methylation status at each individual CpG dinucleotide in the SRR1 and SRR2 region of Sox2, DNA collected at each differentiation

time point was treated with bisulphite, then PCR amplified and sequenced. Unfortunately SRR1 did not yield good quality sequence results and due to limitations of time only the region of SRR2 which gave readable sequence data was further analysed.

The region analysed by sequencing contained three CpG dinucleotide which were found to be completely unmethylated at day-0, day-7, day-14, day-21 and day-28 in both population of cells i.e. control and OS as shown in Figure 3.44 (see section 3.4 for detailed explanation). This data was obtained by direct sequencing of amplified product using DNA prepared from cells collected from two independent biological replicates (sequencing chromatograms are given in Appendix 7.5).

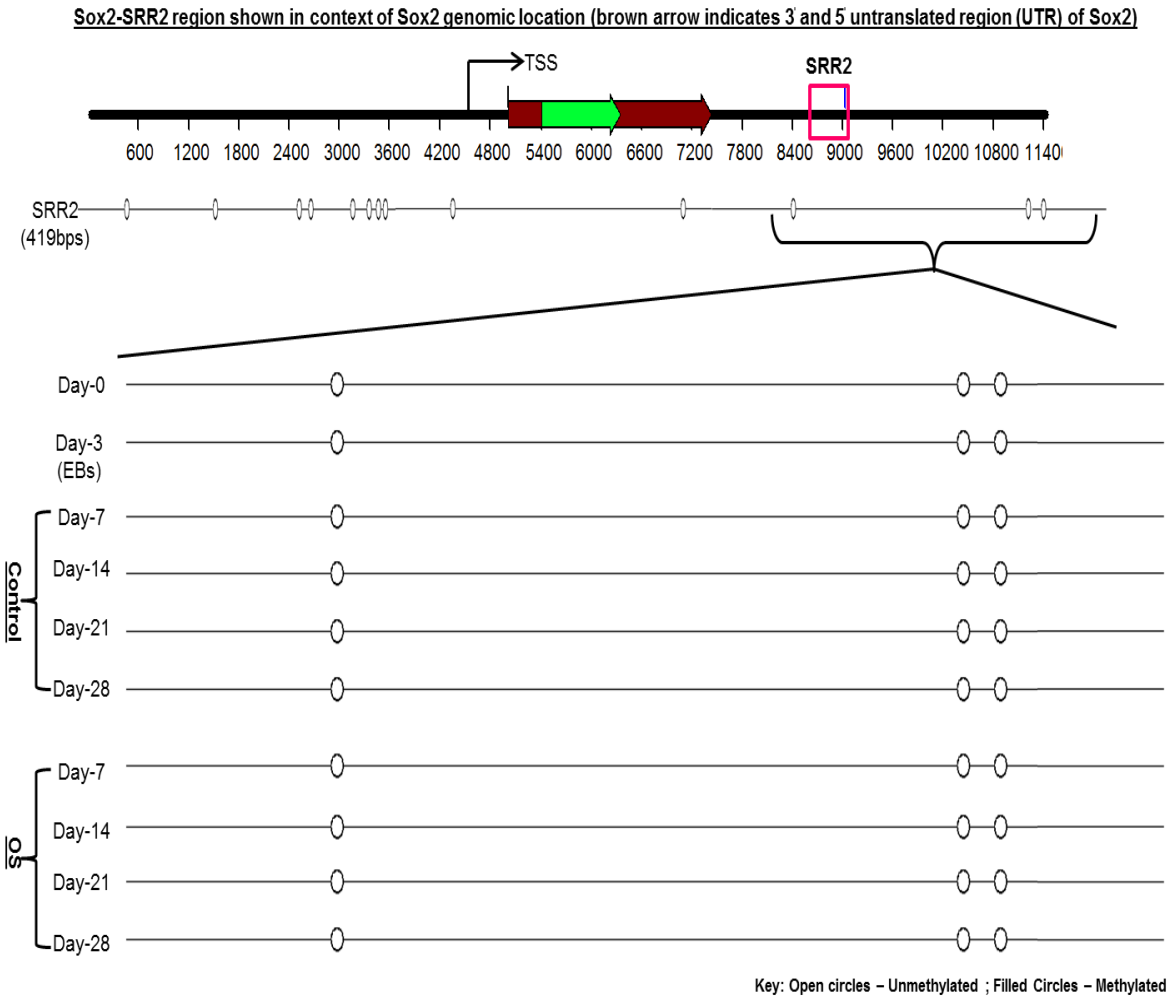


Figure 3.44: Lollipop diagram showing DNA methylation status of CpG dinucleotides in SRR2 region of Sox2. These three sites analysed by direct DNA sequencing of amplified product of SRR2 region showed no methylation at any of time-points chosen for analysis.

3.4. Discussion

The work presented in this chapter described the differentiation of mouse embryonic stem cells (E14Tg2A) into osteoblast though the formation of embryoid bodies in medium supplemented with factors known to induce osteogenesis (Buttery et al., 2001). Different studies attempting in vitro osteogenesis have used mineralization capacity of cells undergoing differentiation and expression of osteoblast specific marker at RNA and protein level to characterize the ososteoblasts (Duplomb et al., 2007).

So the cells grown in osteoblast differentiation medium were first analysed by staining with Alizarin red dye which has been historically used to characterize the calcified mineral depositions in bone matrix (Gregory et al., 2004). The cells grown in osteogenic differentiation medium showed mineralization in a time dependent manner i.e. increased with culture time as is evident in Figure 3.7 (microscopy) and Figure 3.10 (quantitative analysis).

Osteoblast differentiation is a multistep complex process under the control of a myriad of transcription factors and signalling pathways (Lian et al., 2006). The different stages of bone development are proliferation of osteoprogenitors, deposition of extracellular matrix and mineralization (Aubin, 1998). So the expression pattern of markers associated with osteoblasts is organized temporally and sequentially (Nieden et al., 2003). Type I collagen, Alk-P and osteonectin have been reported to appear at the end of proliferation phase; osteopontin followed by bone sialoproten and Runx2 at the start of mineralization phase and finally osteoclacin at high level during mineralization and they can have varying times of onset in culture dependent on cell line and methods of differentiation (Duplomab et al., 2007).

Runt related transcription factor 2 (Runx2 / Cbfa1 / Osf2) is thought to be a central gene co-ordinating signals from BMP and Wnt pathways for cellular commitment and osteogenesis (Lian et al., 2006). It is one of the earlier markers and required for the formation of preosteoblast and Runx2 null mutant have been reported to show no endochondral and membranous bone formation (Komori et al., 2006). Down regulation of Runx2 has been shown to be associated with chondrocyte commitment (Lengner et al., 2005). As reported by earlier studies, Runx2 mRNA was detectable at earlier stages and remained present throughout the differentiation time course peaking at day-14 in OS cells and then down regulating compared to control/non treated cells (Kawaguchi et al., 2005; Hwang et al., 2008). As shown in Figure 3.30, similar pattern of RNA expression was observed in this study where reduction in expression level was seen after day-21 in both OS-treated and non-treated cell populations.

Osterix is osteoblast specific transcription factor acting downstream of Runx2 and lack of Osterix has been shown to associate with no bone formation (Nakashima et al., 2002). It has been shown to be up regulated at later stages of differentiation by an early study (Woll and Bronson, 2006). Here in this experiment no osterix expression was detected at any point of time course either at RNA level or protein level. As cells lacking osterix would not form bone and fail to express osteoblast specific marker genes, it could be possible that PCR reaction was not efficient enough, RNA transcript was not abundant and/or short lived and could not be detected at time points chosen for analysis. As for protein expression, it is possible that antibody used needed further optimization to detect osterix protein since known cell line expressing osterix was not available to use as a control and verify.

Sox9 has been reported to act on Collagen II (col2a1) to ensue chondrogenesis and persistent expression of this has been reported to block osteogenesis (Mori-Akiyama et al., 2003). Here Sox9 expression was found to be induced in cells grown in medium with and without osteogenic supplements at day-7 and RNA observed to be lasted until day-28. Sox9 protein was also observed to be present at these time points with majority of cells positive at day-21 in OS cells and then only few retained expression of that at day-28. It could mean that all of the cells in culture are not undergoing differentiation and some progenitor type cells are still present.

Alkaline phosphatase has been reported to be expressed in large amounts in osteoblasts (Zernik et al., 1990) and is an established marker for mineralization and osteoblast characterization (Benayahu et al., 1989). Also it's an established marker of undifferentiated ES cells (Phillips et al., 2001). Alk-P was found to be peaking up in expression at day-14 in OS cells compared to control and then down regulated as very faint product can be seen at day-28.

Osteopontin is a glycophosphoprotein that binds with calcified matrices to provide adhesion and secreted by osteoblasts at early stage of development (McKee and Nanci, 1996). It has been shown to express at the start of mineralization phase during bone formation and found to be upreglated in cultures upon induction with osteogenic supplements (Nieden et al., 2003; Kawaguchi et al., 2005). Here OPN expression was found to be down regulated after day-21 in OS cells compared to control cells.

Osteocalcin is considered to be an osteoblast-specific gene (Lian et al., 1989) and its expression is associated with matrix synthesis and mineralization (Ryoo et al., 1997; Nieden

et al., 2003). OCN was strongly up regulated after day-14 in OS cells compared to control cells both at RNA level and protein level.

Oct4, Nanog and Sox2 were included as markers of pluripotency and it has been well established now that down regulation of these factors leads to the differentiation of embryonic stem cells (Boyer et al., 2006). Oct 4 is down regulated when stem cells differentiate during gastrulation and subsequently it becomes localized to germ cell lineage (Yeom et al., 1996). Furthermore target gene deletion experiments of Oct4 have resulted in failure of fetal development and differentiation to trophectoderm lieage (Nicholas et al., 1998).

Lack of Nanog in ES cells favours differentiation towards endeoderm lineage (Chambers et al, 2003). Nanog has been reported to inhibit BMP induced mesoderm differentiation of ES cells by interacting with Smad1 pathway (Suzuki et al., 2006). Sox2 has been reported to repress osteoblast differentiation by down regulating Wnt target genes through FGF signalling (Mansukhani et al., 2005). Also Sox2 has been reported to act as repressor of osteopontin gene which is a middle stage marker of osteoblasts (Botquin et al., 1998). Sox2 has been shown to be present in osteoprogenitors stimulating their proliferation over differentiation (Basu-Roy et al., 2010).

As expected all three genes showed significant down regulation upon differentiation at day-3 stage but seemed to be again up regulated after day-21. It is possible that only RNA is present in cells while no functional protein is there. Post transcriptional gene silencing involving long non coding RNAs and small interfering RNAs is now considered important regulatory phenomenon particularly with reference to their role in embryonic stem cell differentiation

(Tay et al., 2008; Dinger et al., 2008). Sox2 protein was seen until day-14 in OS treated cells while no expression was detected at RNA level past day-3 which could be due to transcript having short half-life than protein. It has been shown by some previous studies that mRNA stability is related to molecular function of encoded protein and transcription factor mRNAs are fast degrading transcripts compared to other transcripts (Yang et al., 2003).

Taken together this data suggests that cells are undergoing differentiation as markers associated with osteoblast phenotype are upregulating after two weeks in culture i.e. Sox9, Alk-P, Runx2 and osteocalcin at around the third week with down regulation of pluripotency-related markers particularly, Sox2, after day-14 which would otherwise has been known to favour the maintenance of osteoprogenitors over differentiated cell types. Sox9 expression was seen until day-28 at both RNA and protein level which could mean that not all of the cells are terminally differentiating and some progenitors like cells still exist in culture. Since Sox9 expressing cells can still differentiae to either chondrocytes or osteoblasts. Given the fact that cells are indeed differentiating, it is a good model for looking at down regulation of Sox2 as ES cells differentiate.

Methylation of DNA cytosine residues at the carbon 5 position ($5^{me}C$) is a predominant epigenetic mark in many eukaryotes found in the sequence context of CpG and most often leads to silencing of a gene if found at a promoter sequence. However, increased CpG DNA methylation is associated with gene bodies of actively transcribed regions in mammals and plants (Laird, 2010). Two regulatory regions of Sox2 (SRR1 and SRR2) chosen for DNA methylation analysis were found to be methylated when examined by MS-PCR at all time-points except day-0 and day-3. As this technique only tests the CpGs within Msp restriction

enzyme recognition site, bisulphite sequencing analysis was also carried out to better understand the extent to which methylation occurred within these regions.

Different approaches can be used to study DNA methylation depending on the objective of analysis i.e. genome wide/global or locus specific/single gene (Laird, 2010; Shen and Waterland, 2007). Methylation-sensitive restriction enzymes are primarily used to identify the regions of DNA methylation in chromosome and then depending on goal of analysis either affinity enrichment strategies (MeDIP based) are used or bisulphite conversion is chosen (Laird, 2010). Methylated immunoprecipitation (MeDIP) allows the efficient enrichment of methylated DNA and assay depends on the use of an antibody specific for methylated cytosines to immunocapture methylated genomic fragments (Weber et al., 2005). Methylation profiles obtained by MeDIP approach are not base-pair specific even when combined with arrays (MeDIP-ChIP) or next-generation sequencing (MeDIP-seq) since resolution is restricted by the size of sonicated DNA fragments (Chavez et al., 2010; Laird, 2010). Hence MeDIP based approaches are chosen when the objective is to create genome-wide methylation profiles. Bisulphite conversion and sequencing is the sensitive and preferred method of choice to resolve DNA methylation at single base-pair level (Laird, 2010). This study aimed to screen DNA methylation status at each individual CpG site in SRR1 and SRR2 regions of Sox2, hence methylation-sensitive restriction enzymes were used as a first step to screen for presence or absence of DNA methylation and then bisulphite conversion was used to study each CpG site.

Unfortunately the whole regions of both SRR1 and SRR2 could not be sequenced as direct DNA sequencing attempts after bisulphite treatment were unsuccessful and time limitation did not allow attempting cloning of the fragment and subsequent clonal analysis. Only a part

of SRR2 region could be resolved at individual CpGs which fortunately contains the sequence which is core SRR2 sequence and contains an enhancer site which is under the control of Sox2/Oct4 complex (Tomioka et al., 2002) and has been reported as neural stem cell specific enhancer in telencephalon (Miyagi et al., 2006). Three CpGs located in this enhancer region in SRR2 were analysed by direct sequencing and they all were found to be unmethylated in both undifferentiated and differentiated cells as shown in Figure 3.44. This region has been reported to exert its enhancer activity in embryonic stem cells and neural stem cells but do not function when cells differentiate (Miyagi et al., 2004).

So it would be of interest to profile DNA methylation status of these regions in other differentiation pathways too. This study has also looked into DNA methylation status of SRR2 region in ES cells when they were grown in neural differentiation medium and the next chapter (Chapter 4) summarizes the results of those analyses. This region has not been yet investigated for DNA methylation profiling in differentiating mouse embryonic stem cells particularly when they are grown in osteogenic differentiation cocktail. The data presented here points towards an interesting and important finding that this region could possibly get methylated at other CpG sites in SRR2 than those analysed here as cells undergo differentiation though still preliminary and need further work.

Chapter 4 - DNA methylation analysis of Sox2 regulatory regions during *in vitro* differentiation of mouse embryonic stem cells in neurogenic medium

4.1. Introduction

This chapter summarizes the findings of experiments about mouse embryonic stem cells (E14tg2a) grown in media known to promote neural differentiation.

4.1.1. Neurogenesis

Neurogenesis is an on-going and continuous process in the brain resulting in differentiation, maturation, localization and functional incorporation of new cells into neuronal networks from neural progenitors (Ming and Song, 2005). The adult brain and spinal cord contains a population of stem cells that can generate three major cell types of the central nervous system i.e. astrocytes, oligodendrocytes and neurons and are referred to as neural stem cells - NSCs (Clarke et al., 2000). Neuron, are the functional component of the nervous system and are involved in information processing and transmission, while oligodendrocytes and astrocytes, collectively known as glia, are supportive cells required for nervous system maintenance (Zhao et al., 2008).

NSCs have been shown to be spatially heterogeneous and temporally specified generating progressively restricted cell types (Merkle and Alvarez-Buylla, 2006). The most active regions of an adult brain in terms of neurogenesis are the sub-ventricular zone (SVZ) of the lateral ventricle in the olfactory bulb and subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Duan et al., 2008). Figure 4.1 is a schematic illustration of NSCs localization in adult brain and different cell types originating from them.

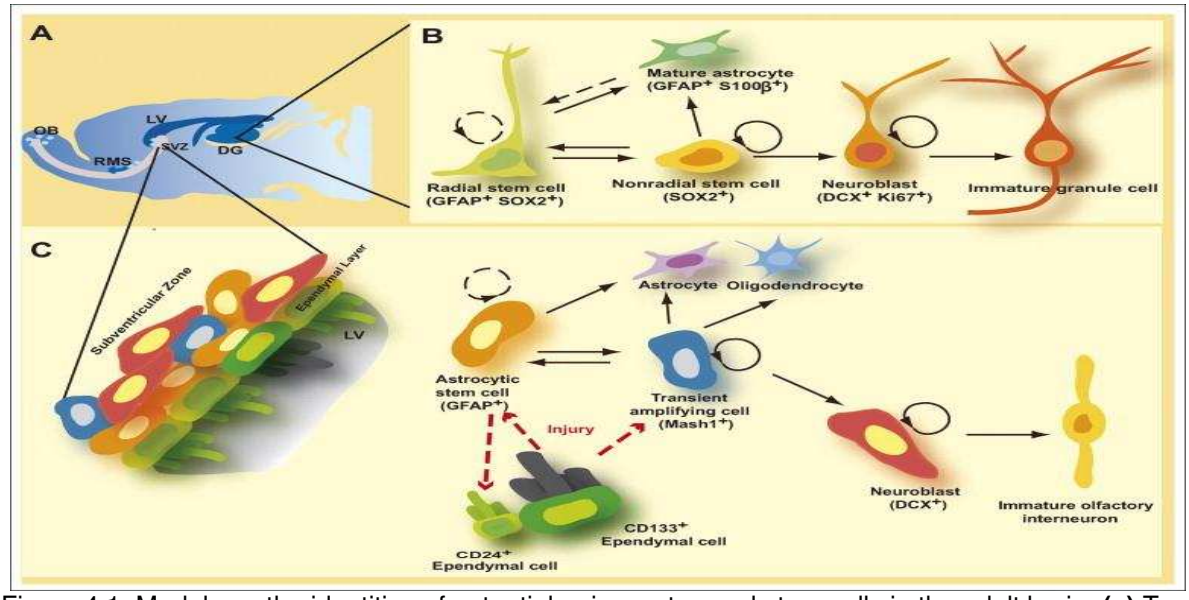


Figure 4.1. Models on the identities of potential quiescent neural stem cells in the adult brain. **(a)** Two neurogenic regions in the adult brain: the subgranular zone (SGZ) in the dentate gyurs (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (LV). **(b)** Potential lineage relationships in the adult SVZ. **(c)** Potential lineage relationships in the adult SGZ (text and figure from Duan *et al*., 2008)

4.1.2. ES cells as a model to study neurogenesis

Neurogenesis is a complex process and much of the current knowledge about vertebrate embryogenesis has been obtained through studies in animal embryos. But the heterogeneity of the cell types involved in neurogenesis and difficulties in obtaining sufficient number of cells for detailed molecular and signalling cascades analysis have limited their regional and temporal characterization (Suter and Karause, 2008). Embryonic stem cells, being harvested from the inner cell mass of embryos and maintaining the multilineage development potential in cultures have become an attractive system to study gene functions and gene regulations during directed differentiation experiments and also understand developmental events (Murrey and Keller, 2008). Hence mechanistic characterization of complex regulatory networks involved in neurogenesis obtained using embryonic stem cell systems would aid in understanding normal brain development and eventually, to develop cell-based therapies for

central nervous system disorders that affect significant sections of population (Schwartz et al., 2008).

ES cells are normally maintained in culture in an undifferentiated state by addition of a cytokine, leukaemia inhibitory factor (LIF) in culture media. When this LIF is removed from the culture medium, ES cells can be made to differentiate into different cell types (Smith, 2001). In the absence of LIF in suspension culture, ES cells form cellular aggregates called embryoid bodies (EBs) which have been shown to recapitulate events of early mammalian embryogenesis and form derivatives of all three germinal layers in vitro (Doetschman et al., 1985; Guan et al., 1999). EBs are initially (within 2-4 days) an outer layer of hypoblast like cells (extra embryonic visceral endoderm) surrounding an epiblast like core (shown schematically in Figure 4.2). At this stage EBs are able to generate derivatives of all three germ layers; definitive endoderm, mesoderm and ectoderm. Between day 6 and 8, the core undergoes cavitation and forms an inner epithelial layer where cells can be committed to definitive ectoderm which subsequently assumes a morphology resembling to neural tube (Coucouvanis and Martin, 1995; Rathjen and Rathjen, 2001; Rajthen et al., 2002).

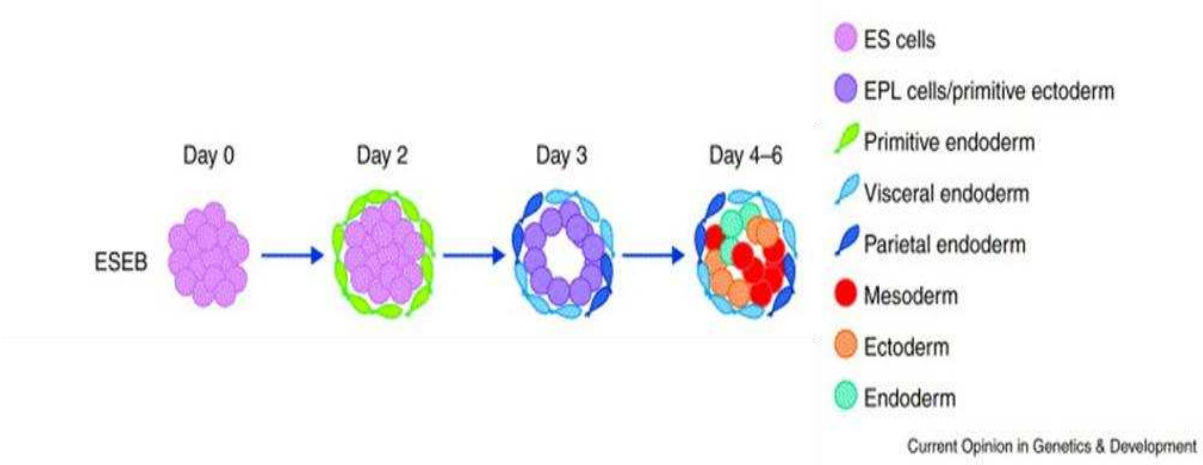


Figure 4.2: Schematic representation of ES cell differentiation into EBs (Rathjen and Rathjen, 2001).

Thus EB mediated differentiations potentially provide a model system for characterization of early embryogenesis and by using varying culture condition various cell types can be generated in vitro by lineage induction and/or lineage selection. However, lack of positional and organizational information coupled with poor characterization of molecular and signalling networks governing the cell fate decisions within EBs also lead to spontaneous differentiation to unwanted cell types resulting in heterogeneous cultures (Nishikawa et al., 2007). This can be overcome by using optimal differentiation protocols using defined media supplemented with specific inductive factors and/or lineage selection if appropriate marker is available to achieve highly enriched desired cell types (Keller, 2005). Non EB approaches of directed ES differentiation in monolayer cultures using serum free, defined culture conditions have also been documented but with little success compared to EB mediated differentiation (Yin et al., 2003; Tada et al., 2005; Yasunaga et al., 2005; Cai et al., 2007). The cell types obtained through these directed differentiation experiments are routinely characterized and analysed by cellular morphology, cell surface markers, gene expression patterns, marker proteins and functional characterization of cell phenotypes (Wobus and Boheler, 2005).

Neural differentiation from ES cells has been documented using variety of protocols. Most of these protocols employ formation of EBs which are enriched using retinoic acid to form neural progenitors which can then be made to differentiate into any of neural lineages: neurons, astrocytes or oligodendrocytes using appropriate culture conditions (Stavridis and Smith, 2003; Cai and Grabel, 2007). Retinoic acid (RA) has been reported to influence nervous system development and maintenance (Maden, 2007) and has been shown to enhance neural gene expression and repress mesodermal specification (Bain et al., 1996). Many differentiation protocols make use of RA at different stages of EB formation in varying concentrations depending on the neural subtype needed (Okada et al., 2004).

Earlier studies reporting the in vitro differentiation of mouse embryonic stem cells to neural cell types applied RA at different time intervals to EBs and then subsequently plated them as intact aggregates in serum containing media using different attachment substrates (Bain et al., 1995; Strubing et al., 1995; Fraichard et al., 1995; Finlay et al., 1996). Later studies combined this approach of initial treatment of EBs with RA, disaggregated and plated them in serum free media where specific growth factors were added to favour differentiation towards a particular neural subtype and have reported to achieve purity up to 90% (Li et al., 1998; Bible et al., 2004; Bible et al., 2007). Neural differentiation has also been reported in monolayer culture without EB formation and RA treatment (Ying et al., 2003). Extent of neural differentiation was studied in every study by observing typical neuronal morphology using phase contrast microscopy, expression of neural specific genes and/or response of those cells to neurotransmitters by electrophysiology.

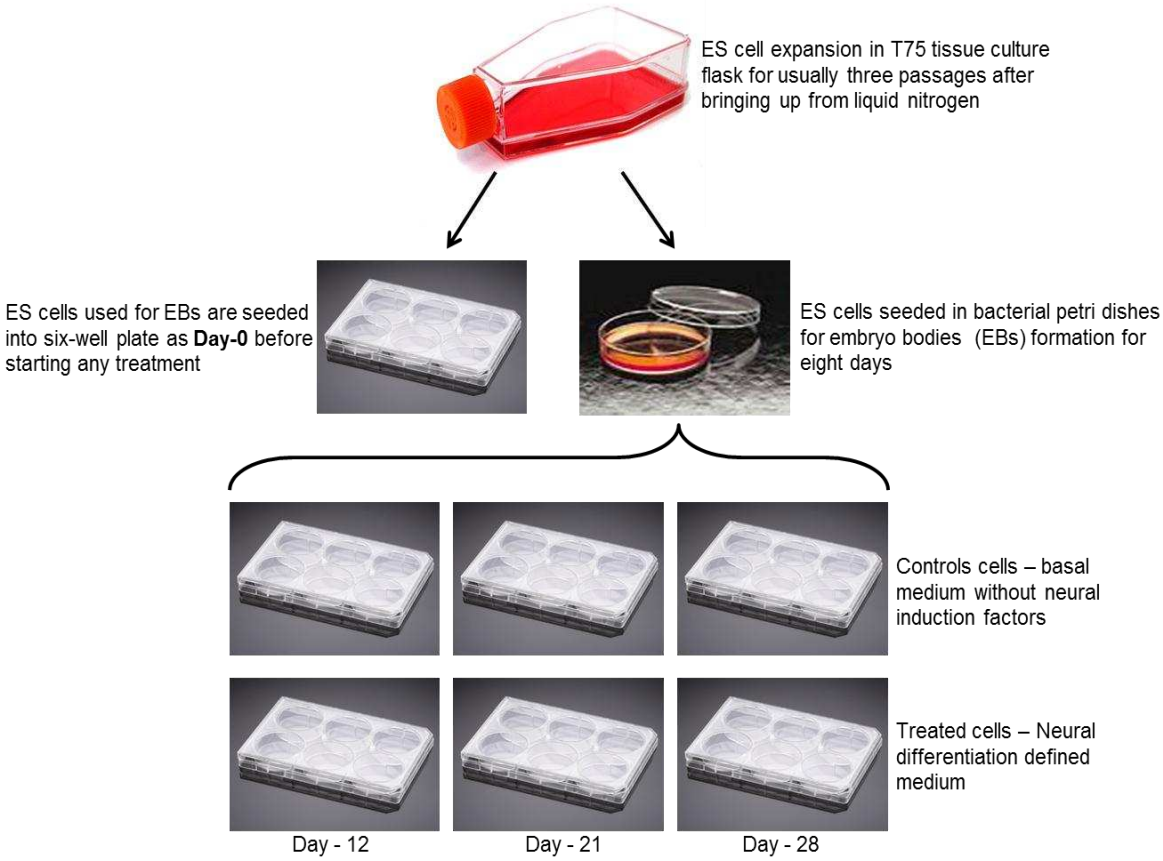
4.1.3. Sox2 in Neurogenesis

A number of transcription factors of the SOX family have been shown to regulate pathways common to all neural stem cells regardless of their origin and state in the brain. They are mainly members of the Sox B1 subclass (Sox1, Sox2, Sox3); Sox E subclass (Sox8, Sox9, Sox10) and Sox B2 subclass (Sox21) (Pevny and Placzek, 2005). Sox2 is reported to be expressed throughout the mouse embryogenesis during initial phases of development and subsequently becomes localized to the neural progenitors (Wood et al., 1999). Loss of function of Sox2 has been shown to promote premature differentiation of neural progenitors to neuronal cell types (Graham et al., 2003). At the same time, high expression of Sox2 has also been found in some post mitotic neurons of the thalamus, medial-dorsal striatum and

septum of adult brain implying its role in neuronal function and maintenance (Ferri et al., 2004).

It has been shown that the expression of Sox2 is regulated by different enhancers during neurogenesis which are temporally and spatially specific and are significantly conserved among vertebrates (Kamachi et al., 2009; Uchikawa et al., 2003). This chapter summarizes the results of experiments aimed to look at DNA methylation changes of a regulatory region of Sox2 (SRR2) in ES cells grown in neural differentiate media. It was hoped that gaining an insight into regulatory mechanisms by which this enhancer act would possibly help to understand neural stem cell maintenance, differentiation and commitment in neurogenesis.

4.2.Experimental work plan



[Cells from each well were then used for subsequent analyses such as Immunocytochemistry (sterile cover slips were placed in the well prior to inoculation of cells, RNA and DNA extraction]

4.3. Results

4.3.1. In vitro Differentiation of mES cells towards neural lineage

E14Tg2a cells were first grown in EB medium for 4 days and then for additional 4 days after adding all-trans-retinoic acid (RA) in medium using this protocol (Bibel et al., 2007). It has been shown that addition of RA during EB formation in media promotes formation of neural progenitors. These EBs were then disaggregated and cells plated in control and neural differentiation medium and, allowed to grow for about 28 days. Cells plated in neural differentiation medium started to show typical spindle shaped neuronal morphology after 24 hours of plating. Neurites were then observed to extend from the cells and grew out over the differentiation time course (some representative pictures in Figure 4.3). By the end a dense network of neurons emerged and found to be distributed throughout the culture surface (Figure 4.3). In contrast, cells plated in control medium failed to grow and beyond day-14 no attached cells were observed in medium. Day-12, 21 and 28 were chosen for analysis (representing second, third and fourth week of culture) but the same analysis was not possible for cells seeded in control medium as no cells were found attached after day-14 (Figure 4.3 shows pictures of cells dying at day-12 and day-14).

4.3.2. Characterization of neural differentiation by Immunocytochemistry

Immunocytochemistry was performed using antibodies against Sox2 and Sox9 to detect the localization and expression of these two proteins associated with neuronal cell types. Almost all of the cells in undifferentiated ES cultures found to express Sox2 (Figure 4.5 day-0) whereas no Sox9 expression was detected (Figure 4.5 day-0). There was no expression of

either Sox2 or Sox9 in cells cultured in neural differentiation medium at day-12, 21 and 28 respectively (Figure 4.5 and 4.6). In order to account for auto fluorescence and/or nonspecific staining cells were fixed and stained in parallel set of experiment but without using primary and secondary antibodies (Figure 4.4).

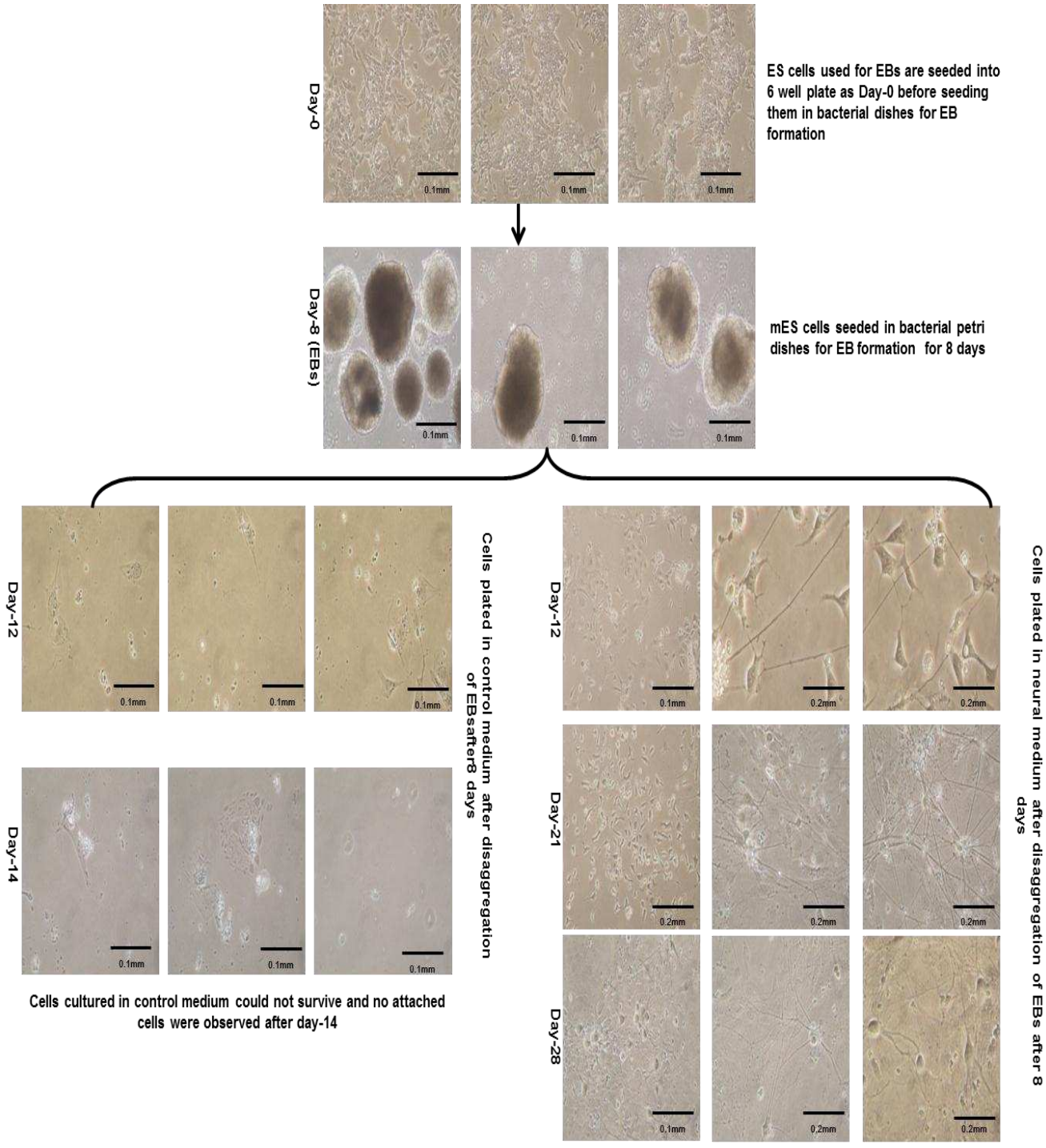


Figure 4.3: Some representative pictures of cells grown in neural differentiation medium at time-points chosen for analysis. mES cells were first expanded in complete ES media and then seeded in bacterial culture dishes for EB formation. Cells were also seeded at this stage in 6 well plate as Day-0 (starting population of ES cells for subsequent biochemical and molecular analysis). EBs were dissociated after 8 days and cells plated in medium containing and lacking neural supplements. Cells subjected to grow in control medium died while those seeded in neural medium grew and showed typical neuronal morphology under microscope.

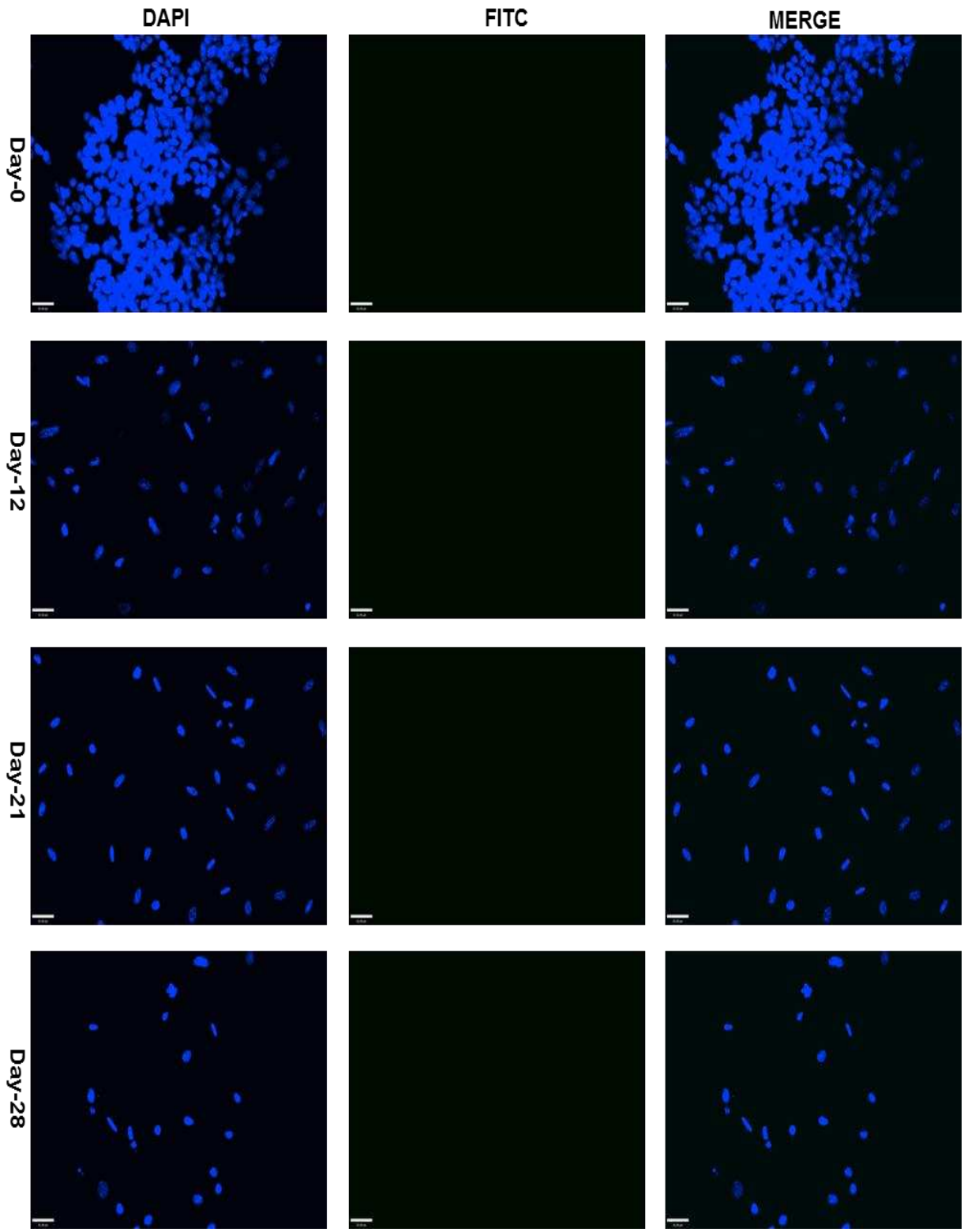


Figure 4.4: Mouse ES (E14tg2a) cells grown in neural medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

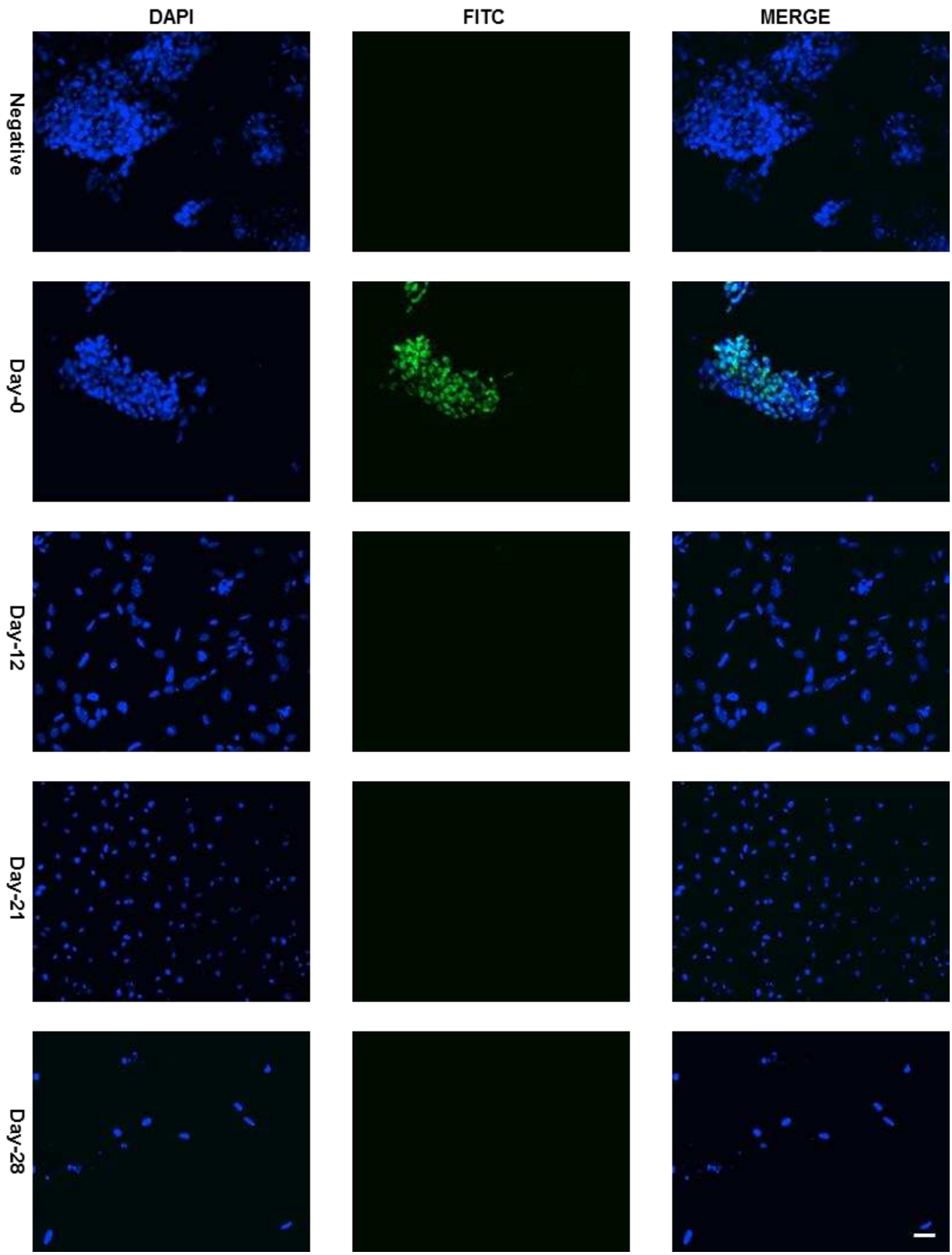


Figure 4.5: Images of E14Tg2a cells undergoing differentiation in neurogenic medium at day-0, 12, 21 and 28 after performing immunofluorescence. Antibody against Sox2 was used to stain the cells and almost cells of undifferentiated ES cell colony were found positive and no expression was detected at day-12, 21 and 28 (Dapi was used to stain nucleus and FITC conjugated secondary antibody to stain Sox2; all pictures were taken at 20X magnification and scale bar is 32µm)

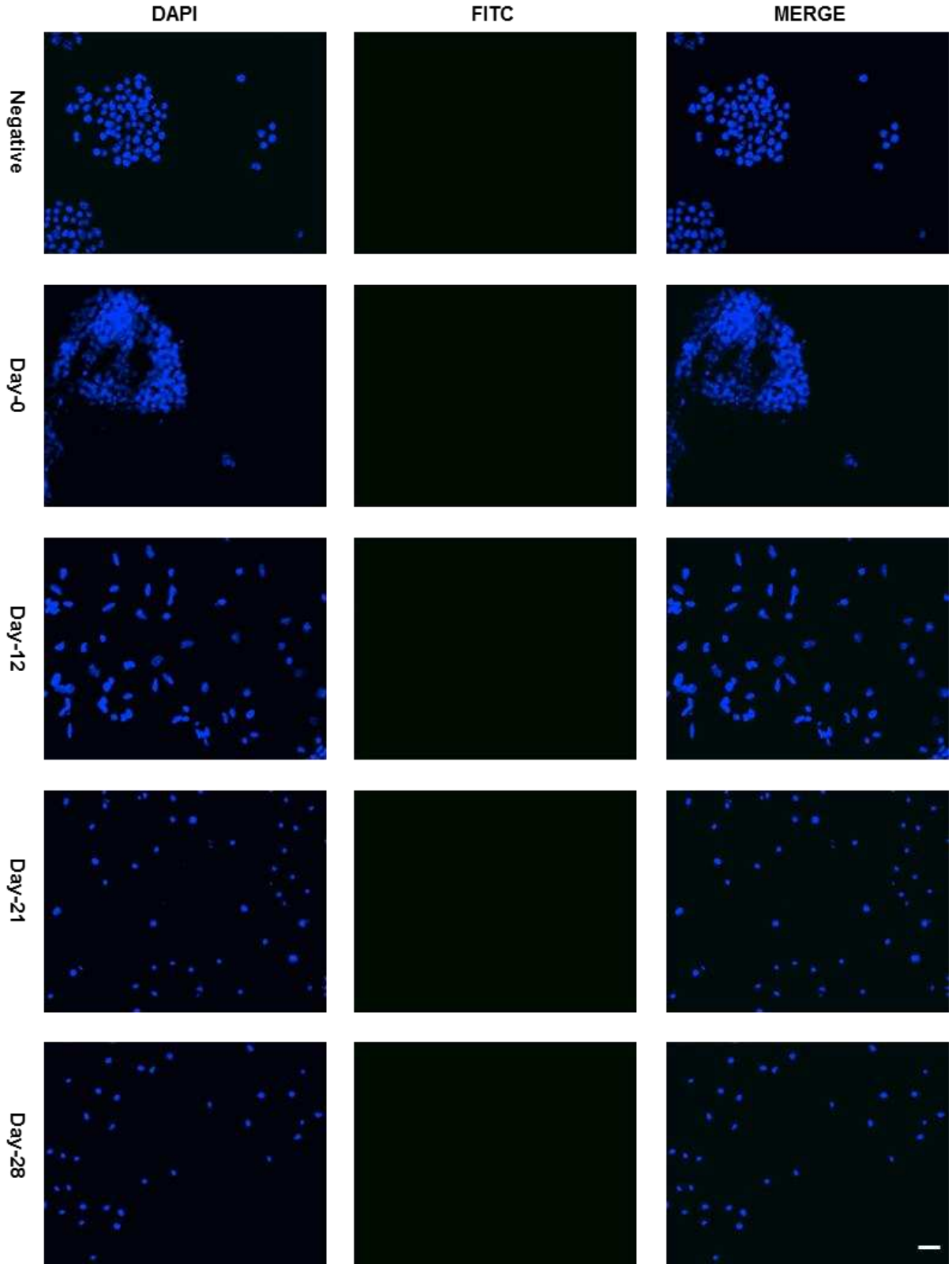


Figure 4.6: Images of E14Tg2a cells undergoing differentiation in neurogenic medium at day-0, 12, 21 and 28 after performing immunofluorescence. Antibody against Sox9 was used to stain the cells and no expression was detected at day-0, 12, 21 and 28 (Dapi was used to stain nucleus and FITC conjugated secondary antibody to stain Sox2; all pictures were taken at 20X magnification and scale bar is 32µm)

4.3.3. Molecular characterization of differentiated cells

4.3.3.1. Gene expression analysis by RT-PCR

Gene expression profiling of embryonic stem cells pluripotency markers Oct4, Nanog, Sox2 and some of neural-markers i.e. Sox9, Nestin, Pax6 and beta-tubulin III were carried out employing random primed cDNAs prepared from RNA extracted from cells undergoing differentiation at different time-points. A house keeping gene GAPDH was used as endogenous control (section 3.3.4.1 for using GAPDH as a normalization control). Products of PCR were visualized by ethidium bromide stained agarose gels using AIDA image analysis software (Figure 4.7) and semi-quantification of gene products was carried out by taking ratio of gel bands to that of GAPDH (Figure 4.8-4.9).

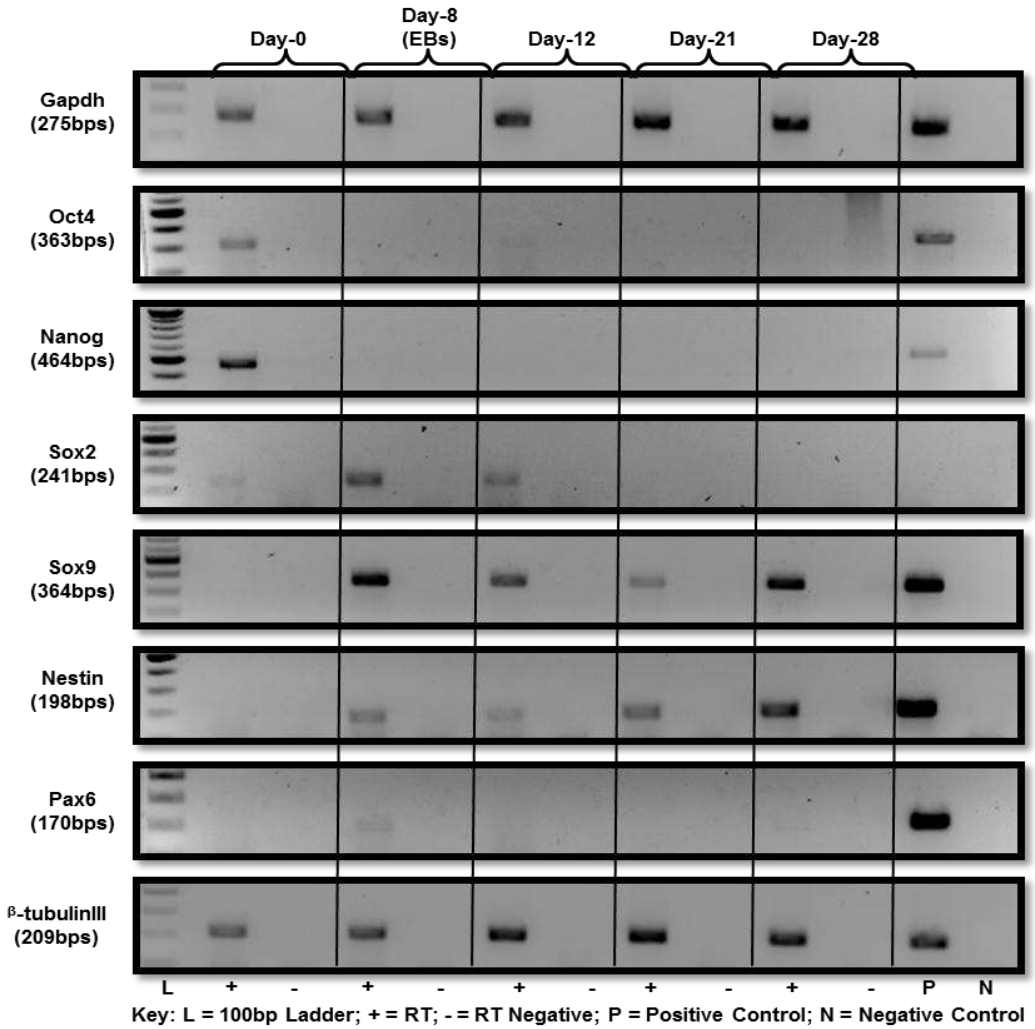


Figure 4.7: Gene expression profiling of mouse ES cells grown in neural differentiation medium using random primed cDNA prepared from cells harvested at day-0, 12, 21 and 28 following differentiation.

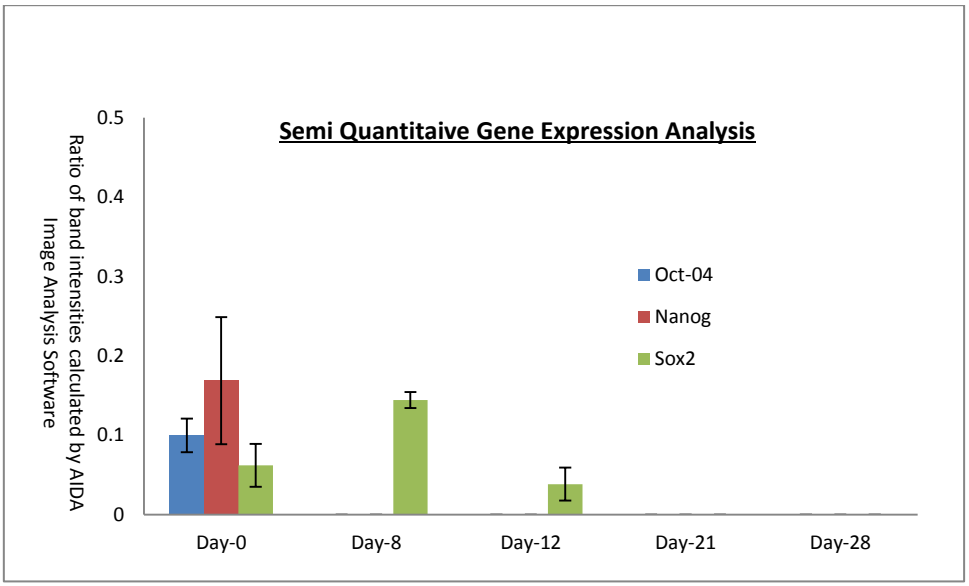


Figure 4.8: Semi-quantitative gene expression analysis showing expression trends of Oct-4, Nanog and Sox9 genes in mouse embryonic stem cells grown in neural differentiation medium. Results are presented as mean±SEM.

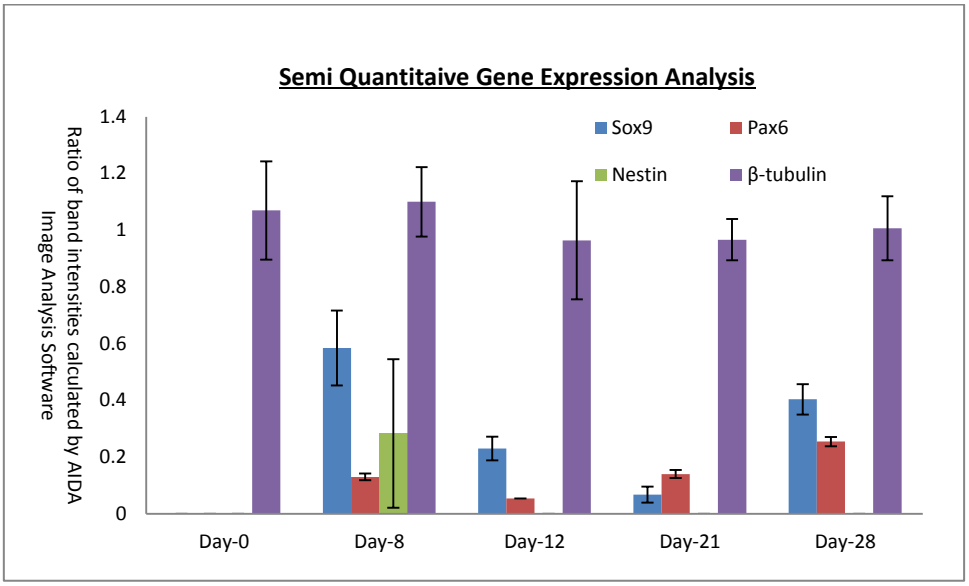


Figure 4.9: Semi-quantitative gene expression analysis showing expression trends of Sox9, Pax6, Nestin and beta-tubulin III genes in mouse embryonic stem cells grown in neural differentiation medium. Results are presented as mean±SEM.

Oct4, Nanog and Sox2 were used as markers of embryonic stem cells pluripotency and all were found to be expressed at day-0. Both Oct4 and Nanog were observed to be down regulated after day-0 as cells differentiate with a very small expression of Oct4 at day12. Sox2 was found to be expressed until day-12 and then beyond that no expression was found at RNA level (Figure 4.7 and 4.8).

Sox9 was observed to be up regulated at day-8 EB stage and then down regulated and seemed to be again up regulated at day-28 stage following plating of dissociated EBs (Figure 4.7 and 4.9).

Nestin was found to be upregulated at day-8 EBs and then found to be expressed until day-28. Pax6 expression was observed at day-8 and then appeared to be down regulated at day-12

post plating of dissociated EBs and no expression was detected at RNA level (Figure 4.7 and 4.9).

Beta tubulin III was included as neural specific marker and found to be expressed at all time-points and apparently peaking in expression at day-12 and lasting until day-28 (Figure 4.7 and 4.9).

4.3.3.2. DNA methylation analysis of SRR2 by methylation sensitive PCR (MS-PCR)

MS-PCR was carried out in the similar way as already detailed in chapter 2 (section 2.3.2) and chapter 3 (section 3.3.4.2). SRR2 region of Sox2 was observed to be methylated at all time-points analysed i.e. day-8, 12, 21 and 28 (Figure 4.10). Controls shown in Figure 4.10 are already detailed in section 3.3.4.2. Upper panel of Figure 4.10 shows the multiplex PCR reaction products for controls where top-band is methylated Igf2R (so band is seen in both Uncut and HpaII lanes), middle-band is DNA sequence lacking any MspI/HpaII sites (band in all three lanes) and lower band is unmethylated sequence of Aprt promoter (only presreent in Uncut lane).

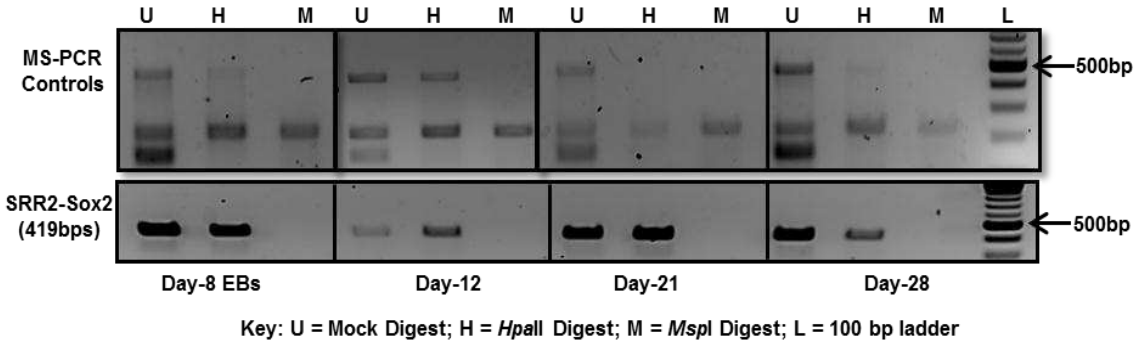


Figure 4.10: MS-PCR gels showing methylated product for Sox2-SRR2 at all time-points chosen for analysis. First the genomic DNA was extracted from ES cells at day-0, 12, 21 and 28, digested using *Msp*I and *Hpa*II restriction enzymes and then analysed by PCR.

4.3.3.3. DNA methylation analysis of SRR2 by Bisulphite Sequencing

In order to further confirm the MS-PCR data and analyse the methylation status at each individual CpG dinucleotide in the SRR2 region of Sox2, DNA collected at each differentiation time point was treated with bisulphite, then PCR amplified and sequenced. The region analysed by direct sequencing contained three CpG dinucleotides that were found to be unmethylated at time-points selected for investigation (Figure 4.11).

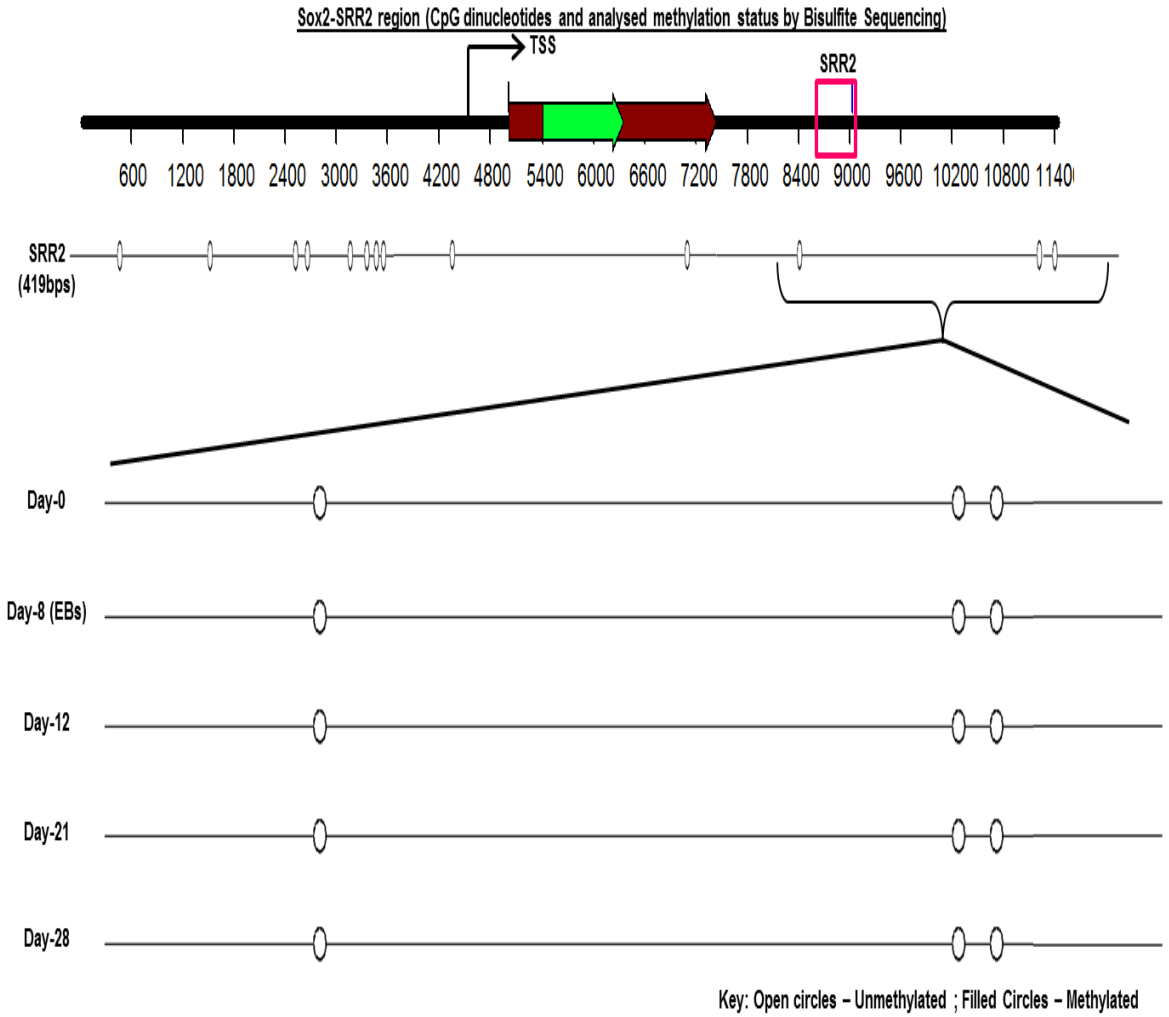


Figure 4.11: Bisulphite sequencing results of DNA methylation status of CpG sites in SRR2 region of Sox2. These three sites were found to be unmethylated in ES cells before differentiation and after culturing for 28 days in differentiation media at all time-points analysed.

4.4. Discussion

Mouse ES cells (E14Tg2a) were first grown in non-adherent bacterial dishes in EB medium to form EBs for 4 days and then retinoic acid (RA) was added to the culture medium and EBs grown for further 4 days. It has been experimentally shown by numerous research studies that a homogenous population of ES cells when treated with RA becomes enriched in neural precursors which subsequently can be differentiated to different neuronal subtypes using various inductive signals and selective media (Bible et al., 2004; Bible et al., 2007; Bain et al., 1995; Strubing et al., 1995; Fraichard et al., 1995; Finlay et al., 1996; Okabe et al., 1996; Li et al., 1998; Stavridis and Smith, 2003; Cai and Geabel, 2007). EBs obtained after 8 days were disaggregated by trypsinization and plated in neural differentiation medium (DMEM/F12 supplemented with neural specific supplement N2) in gelatine coated plates. Cells obtained from the same EB pool were also plated in medium lacking N2 with the objective to use this cell population as control since this was the only additional component to basal medium that could be omitted in parallel set of experiments if they were to use as control in spirit. These EB derived cells when grown in defined medium suffers from a significant reduction in cell number at first since they are transferred from serum-containing medium to serum-free medium, but those that survive are mostly neural progenitors which can be expanded in culture and induced to differentiate into neurons (Bible et al., 2004; Bible et al., 2007; Okabe et al., 1996; Li et al., 1998; Stavridis and Smith, 2003; Ying and Smith, 2003).

Neural differentiation of E14tg2a was first analysed by examining the specific neural morphology under microscope and this was evident after 24 hours of plating dissociated EBs. By day-4 after plating neurites can be seen forming dense and tangled networks with typical

neuronal morphology in N2 media as shown in Figure 4.3 which became more denser with time in culture (by second and third week). This was consistent with the findings reported by studies from which this protocol was derived (Bible et al., 2004; Bible et al., 2007). In the control medium, as expected, very few cells were found to attach 4-days after plating with a morphology resembling that of cells undergoing senescence and by day-6 almost all of the cells had died (Figure 4.3 presents some representative pictures). Cell death could be attributed to the fact that DMEM/F12 is a basal medium (Dulbeco and Freeman, 1959; Ham, 1965; complete composition Invitrogen Gibco 21331-020) and is unable to support growth of cells at its own unless supplemented with further additional supplements that aid in cell attachment, survival, proliferation and/or differentiation (Butler and Jenkins, 1989). And further augmenting the cell death was transfer of cells from serum-based medium to serum-free medium which on top of that also lacked any growth factors to support growth of cells (Ying and Smith, 2003). And this could possibly by more exasperated by pre-treatment of cells to become neural progenitors which also needed specialized environment to grow and expand which was only provided in parallel culture medium supplemented with N2. Since cells seeded in control media all died, this merits explanation what is considered control in such differentiation studies.

As detailed earlier in section 4.1.2 that for differentiation, ES cells are allowed to aggregate in the absence of LIF in suspension culture to form EBs for a defined time periods (in most cases 3-8 days), these EBs are then returned to serum-containing medium or serum-free medium either as aggregates or disaggregated to single cells, and allowed to further differentiate to produce terminally differentiated cell types. Most of the ES cell derived differentiations have been optimized using serum-containing media. Serum is added in cell culture medium as an essential growth supplement where it serves a source of broad range of

macromolecules, hormones, growth factors and provides attachment and spreading factors. But undefined composition of serum, batch to batch variations which often lead to phenotypic differences in cell cultures and risk of spreading contamination are major drawbacks and hence studies are now focusing more towards the use of serum-free media (Bruner et al., 2010; Keller, 2005). In the absence of serum, a large number of molecules need to be added in media that could maintain cell adhesion, proliferation, growth and survival. Defined media are optimized through empirical approaches such that growth of particular cell types is favoured over others. When cells are transferred to serum-free media, it takes some time for cells to adapt to this new environment particularly if they were originally maintained in serum-containing media (van der Valk et al., 2010). One way to overcome this is to seed cells in higher number, so here in this study (described in this chapter) cell seeding density used was adjusted according to original protocol keeping the same surface area to volume ratio as was optimized by them (Bible et al., 2007; Ying and Smith, 2003)

Those differentiation studies that make use of serum-containing medium post EB stage use additional supplements to promote lineage induction/enrichment towards desired cell type. Because additional supplements are added in normal serum-containing basal medium, this necessitates setting up a parallel set of experiments using same EB derived cells under similar conditions but not receiving additional supplements which are then allowed to grow alongside the cells receiving additional supplements. Differentiation endpoint or enrichment of desired cell type is always studied using lineage specific markers (RNA, protein) and functional assaying of cells phenotype. Ultimate test of differentiated phenotype is achieved by transplanting these cells to appropriate animals models where they should retain their functionality (Keller, 2005; Wobus and Boheler, 2005). The idea behind setting up of these parallel set of experiments is to compare the enrichment of particular cell type in response to

added supplements compared to differentiation observed in serum only since serum itself contains inducing factors (undefined composition) as discussed above and is prone to batch to batch variations. In fact the pioneering study that demonstrated the differentiation potential of ES cells into derivative of all three germ layers when they removed the feeder layer maintaining undifferentiated state of cells in standard serum containing media did not use any additional factors (Doetschman et al., 1985). These control culture does not imply that they will have cell types of interest only or will not have cell types of interest at all. These are mixed cell populations derived from same EB pool from where the differentiated cell types of interest are generated.

Traditionally cell populations grown in serum only medium where no additional supplements are added are called "controls" presumably because this is the only constant factor in such experiments while the additional supplements (dose, duration, combination, time of application) added is experimental variable which can be altered according to needs/research question. It should be noted here that this additional control group only serves to compare enrichment of desired/required cell type in response to added growth factors in serum compared to differentiation seen in serum only medium without addition of exogenous factors and is not a control per se. Differentiated cells are always analysed and characterized using features inherent to that particular cell type e.g. morphology, lineage specific markers, functional assessment of phenotype etc. Numerous studies that attempted directed differentiation of ES cells to other lineages using routine serum based media supplemented with some exogenous factors documented their findings in both serum only media and media supplemented with additional factors (an examples is chapter 3 of this study; few other examples are Buttery et al., 2001; Hamazaki et al., 2002).

In contrast studies that use serum-free defined media for directed differentiation towards cell type of interest make use of selective nature of medium to specifically allow growth of desired cell types and hence do not need a de facto control group. As detailed above that such a control group is technically needed in studies that are attempting to achieve differentiation in response to added supplements to routine serum based media so as to compare the differentiation seen with and without applied experimental treatment. But here in these studies using defined media, medium composition is optimized such that only cells able to respond to those signals can survive hence acting as filter in its own against unwanted cell types and is the only experimental variable that can be altered where by definition, control is something that is fixed/same in all experimental conditions tested.

For neural differentiation such protocols have become available which make use of defined media and hence not needing an inclusion of parallel set of experiments in contrast to differentiation studies making use of supplements in routine serum based media. As noted above that this control group only serves to compare the differentiation achieved under experimental conditions tested to what seen in serum only. Differentiated phenotype is always characterized by analysing the cell types generated. With use of defined media compositions such comparison is essentially not needed. Hence those preliminary studies that are aiming to screen for such defined milieu to get terminally differentiated cell types of interest use empirical combinations of different factors known form previous studies to determine the optimal media compositions. For example a study by Okabe et al., in 1996 reported use of serum-free defined media for in vitro neural differentiation of mouse embryonic stem cells. This study aggregated ES cells as EBs for 4 days and then plated those aggregates in DMEM/F12 supplemented with insulin, transferrin, selenium and fibronectin (ITSFn) which was previously reported to be used for neural induction of embryonal

carcinoma cell line (Rizzino and Growley, 1990). They documented significant loss of cell viability at first when cultures were transferred from serum containing media to serum free formulation but ultimately they were able to get neural progenitors where more than 85% were nestin positive. Nestin has been a traditional marker associated with neural precursors that can subsequently give rise to neurons, glia and astrocytes (Landhal et al., 1990). These nestin positive cells were then subsequently differentiated to neuronal cell types with high efficiency using defined medium and differentiated cells were analysed by expression of lineage specific markers and electrophysiology. This study did not include an explicit control rather compared data obtained across different experimental conditions tested to conclude which was optimal.

The principal objective of directed differentiation experiments is to identify conditions that would ideally favour the induction of particular lineage precursors (in EBs or monolayers of ES cells) and then use optimal selective media conditions to allow those precursors to proliferate and generate the pure cultures of terminally differentiated cell types. So eventually desired cell types can be produced in vitro under controlled conditions using defined media supplemented with inductive growth factors (Smith, 2001). For neural differentiation, which is one of the extensive studied in vitro differentiation, such two-step protocols are available. The study presented in this thesis used similar two step protocol of neural differentiation i.e. enrichment of neural progenitors in EBs using RA treatment and then selectively allowing neural precursors to proliferate in defined medium (DMEM/F12/N2) to differentiate towards neural cell types (Bible et al., 2007; Bible et al., 2004). Different studies have shown experimentally that a homogenous population of ES cells when treated with RA becomes enriched in neural precursors which subsequently can be differentiated to different neuronal subtypes using various inductive signals and selective media (Bible et al., 2004; Bible et al.,

2007; Li et al., 1998; Stavridis and Smith, 2003; Cai and Grabel, 2007). And one study has experimentally demonstrated that neural precursors generated by RA treatment (similar to one used in this protocol) can form cells of motoneurons and interneurons in anterior spinal cord when transplanted to embryonic chick neural tube showing that such a treatment could lead to cells which retain functionality when transplanted (Plachta et al., 2004). As detailed above that such a study design which is biased towards enrichment of particular desired cell type using selective treatment does not have a bona fide control. And those studies from which this protocol was derived also did not include such an explicit control in their studies (Bible et al., 2004; Bible et al., 2007). Yet study documented in this chapter attempted to set up a parallel set of experiments as explicit control with the objective to compare differentiation seen with N2 and without N2 since N2 was the only additional component added to basal media. EBs grown for 8 days in suspension culture were disintegrated (section 2.2.4) and seeded in differentiation media (DMEM/F12/N2) and control media (DMEM/F12 alone). The idea to seed cells in DMEM/F12 was to use this group of cells as control but cells did not survive and reasons are discussed above. This step experimentally demonstrated that DMEM/F12 alone did not support growth of cells unless supplemented with additional growth factors/serum.

The experimental set up described in this chapter is different to one described in Chapter 3 (osteoblast differentiation) where differentiation was attempted by adding exogenous factors to serum based medium so control cultures were set up using supplements minus medium. This control contained the same pool of EB derived cells but not received additional supplements. While in neural differentiation experiment only additional supplement added in medium was N2 and so the only way to set up control cultures was to grow cells without N2. Such a step was redundant given that cells do not survive in DMEM/F12 alone as discussed

above. The other way to set up parallel control culture could possibly be using cells grown in absence of RA. But there are already studies available that attempted successful in vitro neural differentiation of mouse ES cells without RA treatment (Okabe et al., 1996; Lee et al., 2000; Rolletschek et al., 2001). They all used specific growth factors in combination with serum-free defined medium and achieved differentiation to different types of neurons. Hence if such a step was included, it would at best served as an alternate strategy to achieve differentiation and not a control culture. The reason why probably neural cell types can be achieved in culture with and without RA is that neural induction is considered a default differentiation pathway during vertebrate embryogenesis under repressive control of BMP signalling (Munoz-Sunjuan and Brivanlou, 2002). Studies have very early on reported that if ES cells are allowed to differentiate under chemically defined serum-free conditions they preferentially express markers of neuroectoderm without addition of any additional growth factors (Wiles and Johansson, 1999). The problem however is that differentiated culture almost always contain different types of neural cells i.e. neurons, astrocytes, oligodendorcytes and glia and that's why variety of protocols are available to achieve enrichment of a particular cell type by addition of specific growth factors at different stage of differentiation subsequent to neural progenitors formation which is a common step in majority of differentiation protocols (Stavridis and Smith, 2003).

Whether there is an explicit control group or not, cells grown in differentiated media are always analysed and characterized by using established markers and functional assays for such cell types. In order to further characterize the differentiated cells beside morphology, a set of markers was selected for expression studies at RNA and protein level in accordance with the data reported for earlier studies reporting in vitro differentiation of embryonic stem cells in to neural lineage (Bain et al., 1995; Bible et al., 2004; Bible et al., 2007; Stavridis

and Smith, 2003). This included Sox2, Sox9, Nestin, Pax6 and beta-tubulin III. Sox2 along with other members of SoxB family has been reported to regulate and co-ordinate neuroectodermal specification and commitment (Pevny and Placzeck, 2005). More specifically, Sox2 has been reported to maintain neural progenitor identity (Graham et al., 2003) with subsequent down regulation as these cells become committed. Here in this experiment Sox2 was found to be down regulated at day-8 EBs stage and then further reduction in expression level at day-12 and beyond that time point no expression was detected (Figure 4.7).

In order to confirm the molecular expression data, protein expression of Sox2 was also analysed by immunocytochemistry and only undifferentiated ES cells were found to be positive (Figure 4.5). No Sox2 protein expression was observed at day-12 which could be due to cells still containing RNA but not transcribing it to functional protein product. Post transcriptional gene silencing involving long non coding RNAs and small interfering RNAs is now considered important regulatory phenomenon particularly with reference to their role in embryonic stem cell differentiation (Tay et al., 2008; Dinger et al., 2008). Indeed, Sox2 gene has been shown to embedded within an intron of long non-coding RNA which transcribes in the same orientation and has been documented to have regulatory roles during vertebrate development (Amaral et al., 2009).

Sox9 showed marked up regulation at day-8 EB stage at RNA level (Figure 4.7) and it has been reported to be up regulated prior to gliogenesis shifting potential of neural progenitors from neurogenic to gliogenic and disappears from oligodendrocyte lineage once cells differentiate terminally (Stolt et al., 2003). It has been shown that retinoic acid treated ES cells form precursors cells with the characteristics of radial glial cells which eventually

differentiate into glutamatergic neurons (Bibel et al., 2007). Sox9 was down regulated after day-8 EB stage and seemed to be up regulated at day-28 after plating of cells in neural differentiation medium. However, no Sox9 protein expression was detected at any time point along the whole differentiation time course starting from day-0 to day-28 (Figure 4.6). As described earlier, microRNAs are known to play an important role in gene regulation and an earlier study has reported the role of miR-124 in Sox9 protein suppression but mRNA expression is unaffected in adult neurogenesis (Cheng et al., 2009).

Nestin is a neuroepithelial stem cell intermediate filament protein and, a well-established marker of neural stem cells and has been widely employed as marker in studies exploring neural differentiation of ES cells (Landhal et al., 1990; Yaworsky and Kappan, 1999). It has been considered as a mutipotent neural lineage marker expressed both in neurons and glia (Lenka et al., 2002). It has been shown to be expressed in developing nervous system, neural stem cells from sub ventricular zone and to become down regulated when the cells differentiate (Wei et al., 2002). Here in these studies nestin was observed to be up regulated at day-8 (EBs) stage and found to be expressed until day-28. As protein expression studies were not carried out, mRNA expression at this late stage could possibly mean that RNA not transcribed into functional protein through mechanisms similar to those described above. Alternatively, this expression may be accounted for by a subpopulation of progenitor cells still present in culture.

Pax6 has been reported to play an important role in neural precursor's proliferation, cortical lamination, and development of basal ganglia, neuronal survival and neurogenic fate determination (Kallur et al., 2008). Pax6 has been shown to undergo dynamic changes in expression as ES cells become committed to neuronal differentiation (Gao et al., 2011).

mRNA expression analysis in these experiments showed that Pax6 is up regulated in EBs after 8 days of differentiation which should be rich in neural precursors after RA treatment. Beyond day-8 no Pax6 expression was detected at RNA level (Figure 4.6). Earlier studies have shown that Pax6 mRNA was not detectable after day-12 in ES cells differentiated to neuronal cell types under similar conditions (Bible et al., 2007; Bible et al., 2004).

Beta-tubulin III belongs to the tubulin class of proteins and shows expression specifically in post mitotic neurons (Lee et al., 1990), it is a classic marker to assess neural differentiation of embryonic stem cells (Bain et al., 1995). In this study beta tubulin III was found to be expressed starting from day-0. It has been shown that undifferentiated mouse ES cells express beta III tubulin (Ginis et al., 2004) though their role in undifferentiated cells has not been studied so far. Beta III tubulin was found be markedly upregulated after day-8 of differentiation and expression lasted until day-28.

Taken together these morphological and molecular data shows that ES are differentiating in this neurogenic media and results obtained are comparable to studies reported earlier. All of the markers associated with neural progenitor's identity tested in this study i.e. Sox2, Sox9, Pax6 and Nestin were found to be upregulated in day-8 EBs compared to no expression at day-0 undifferentiated cells. Then there appeared to be visible upregulation of beta-tubulin III, a marker extensively used to characterize differentiated neural cells. But it must be emphasized that no attempt to ascertain the purity of differentiated culture was made, hence it cannot be said with certainty that how much of differentiated population were neurons. Future work would be targeted to purify the differentiated cells and electrophysiology measurements would be carried out to determine whether neurons are actually forming active synaptic connections. It should also be noted here that the primary objective of the study

presented in this thesis was to study DNA methylation changes occurring in regulatory regions of Sox2 during differentiation of mouse ES cells when they are differentiated under different culture conditions. Hence differentiation was attempted using previously reported optimized protocols and no attempt was made to modify/refine them at this stage of analysis. But in future efforts would be made to compare and contrast different differentiation strategies to select the optimal one when repeating this experiment.

Epigenetic mechanisms of gene regulation involving DNA methylation, histone modifications and regulatory RNAs have now started to emerge as important mechanisms governing the neural specification of neural stem cells (Sanosaka et al., 2010). The information about different regulatory regions of Sox2 and rationale for doing DNA methylation analysis has already been detailed in Chapter 1 section 1.5.3 and 1.6. Different regulatory regions of Sox2 have been implicated in directing region specific expression in cells of differing origin e.g. expression can be seen in telencephalon but not spinal cord stem cells (Zappone et al., 2000).

SRR2, a regulatory region of Sox2 was found to be constitutively methylated at all time-points in the differentiated population studied in this chapter when analysed by MS-PCR. Given the limitation of the technique to resolve methylation status at each individual CpG, bisulphite sequencing was then carried out (section 3.4 for discussion about various techniques to study DNA methylation) . Unfortunately the whole region of SRR2 could not be sequenced as direct sequencing attempts were unsuccessful and time limitation did not allow attempting cloning of the fragment and subsequent clonal analysis. But fortunately analysed region contains the sequence which is core SRR2 sequence and contains an enhancer site which is under the control of Sox2/Oct4 complex (Tomioka et al., 2002). This

enhancer has been reported as neural stem cell specific in telencephalon region (Miyagi et al., 2006).

Three CpGs located in this enhancer region in SRR2 were analysed by direct DNA sequencing after bisulphite conversion of genomic DNA and they all were found to be unmethylated in undifferentiated and differentiated cells as shown in Figure 4.8. It has been shown by another study that this region exerts its enhancer activity in embryonic stem cells and neural stem cells but do not function when cells differentiate (Miyagi et al., 2004). Furthermore an earlier study has shown the differential methylation at SRR2 in neurons v/s astrocytes generated from human neural precursors suggesting that terminally differentiated neurons use methylation of this region to silence the gene (Sikorska et al., 2008). Here no methylation was observed at analysed three CpG sites found in the enhancer region suggesting possible involvement of other gene silencing mechanisms independent of DNA methylation such as histone modifications. Also there is possibility that heterogeneous nature of culture did mask the low level of methylation. So in future experiments effort would be focused towards establishing a pure population of cell types of interest. It could also be possible that other CpG sites not analysed in this study could have become methylated during differentiation. Hence further work extending the analysis to all CpG sites and also investigating chromatin structure surrounding the region would help in understanding the role of this region in gene silencing once cells become committed and differentiate.

Chapter 5 - DNA methylation analysis of Sox2 regulatory regions SRR1 and SRRR2 in mouse mesenchymal stem cells (MSCs) cultured in osteoblast differentiation medium

5.1. Introduction

5.1.1. Mesenchymal stem cells (MSCs) as a model to study osteogenesis

Mesenchymal stem cells are mainly derived from bone marrow though have been shown to exist in many other tissues such as hair follicles, muscles, liver etc. (Bianco et al., 2008). Bone marrow derived mesenchymal stem cells or also known as stromal stem cells; they are considered to be self-renewing and having multilineage differentiation potential forming different mesoderm derivatives including bones, cartilage and adipose tissues (Pittenger et al., 1999). Recently bone marrow derived MSCs been also shown to transdifferentiate into other lineages derived from both ectoderm and endoderm such as neurons and hepatocytes (Charbord, 2010). The most salient features of MSCs are their readiness to grow in culture; as they have displayed and suppression of immune response in vivo; MSCs have already been in use clinically to assess the safety and efficiency of bone marrow transplants in human subjects (Ghannam et al., 2010).

Bone tissue engineering has become an active focus of research due to increased number of people being affected with osteodegenrative disorders including but not limited to osteoporosis, osteoarthritis, skeletal deficiencies (Heng et al., 2004). Thus in vitro differentiation of MSCs into bone tissues in a variety of experimental systems have made them a promising system to be used in cell based therapies for bone tissue engineering and regenerative medicine (Meijer et al., 2007). Besides being used for therapies, they offer a model system to study osteogensis which is under complex control of myriads of molecular mechanisms and signalling networks (Lian et al., 2006).

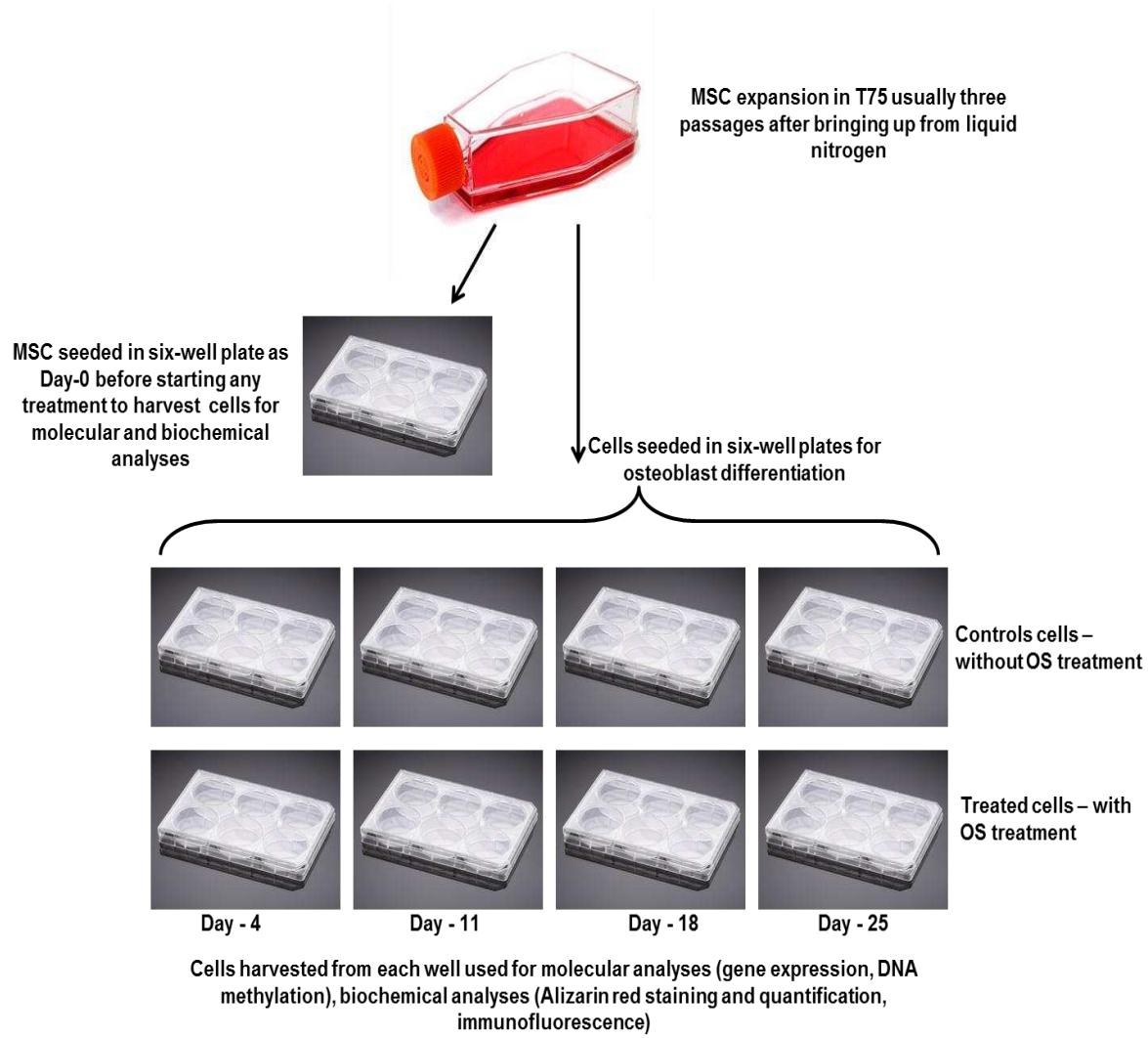
However, the low frequency of MSCs in bone marrow and limited self-renewal capacity has created a need to explore and exploit differentiation potential of pluripotent ES cells into bones which have been shown to regenerate themselves indefinitely under proper culture conditions (D'Ippolito et al., 1999; Duplomb et al., 2007). Furthermore bone formation has been thought to involve a hierarchy of precursor populations existing at various stages of bone development starting mainly from mesenchymal stem cells and going through osteoprogenitors, preosteoblasts and osteoblasts though their well-defined boundaries and compartmentalization are still under investigation (Long, 2001). It has been proposed that MSCs are osteoblastic precursors or pre-engaged cells originating from a primitive progenitor and hence might not allow investigating the early stages of bone development where ES could potentially overcome this issue (Duplomb et al., 2007; Long, 2001). Nevertheless, MSCs have been extensively investigated in vitro for bone formation (section 3.1.1 in chapter 3 for detailed description of osteogenesis), so can also serve as positive control for ES derived osteoblast differentiation.

5.1.2. Sox2 in MSCs

There are only limited studies so far investigating the role of Sox2 in MSCs in general and in osteoblasts in particular. Sox2 has been shown to maintain expansion and differentiation potential of MSCs (Go et al., 2008). Though MSCs can renew themselves and have been used to repair bone disorders (Horwitz et al., 1999), but with time they tend to lose their differentiation potential (D'Ippolito et al., 1999). Hence understating the regulation of Sox2 in MSCs and during their differentiation would aid in refining methodologies attempting to use these cells for therapeutic purposes.

The specific role of Sox2 in osteoblasts is already described in chapter 3 section 3.1.3. In short, data available to date has shown Sox2 to be present in osteoprogenitors favouring their proliferation their over terminal differentiation to osteoblasts. Given that MSCs are physiological precursors of osteoblasts, they were differentiated to osteoblasts in parallel with mouse ES cells using the same protocol and other experimental factors. The aim of these experiments was to study the molecular and biochemical changes occurring in both cell types when they are differentiated using identical experimental conditions. It was hoped that data obtained by this study could potentially help in understanding the regulatory role played by Sox2 in bone development, and conserved/shared gene regulatory pathways between these cell types.

5.2.Experimental work plan



5.3. Results

5.3.1. Differentiation of mMSCs (D1 cell line) into osteoblasts

5.3.1.1. Cell Culture

D1 – a mouse mesenchymal stem cell line (Diduch et al., 1993) was retrieved from liquid nitrogen and cultured on tissue culture treated flasks with routine passaging on confluence to generate enough number of cells to set up differentiation and for cryopreservation. Some representative images of D1 stem cell line are shown in Figure 5.1.

MSCs cells were first seeded in six-well tissue culture plates in an undifferentiated state and allowed to reach confluence before changing to medium containing OS supplements as described in chapter 2 section 2.1.5. Cells grown in medium lacking OS supplements were taken as control. It has been reported that MSCs grown in presence of OS supplements form colonies with mineral deposition which can be further confirmed by microscopy and Alizarin Red staining. As shown in Figure 5.2, the D1 cell line when cultured under similar conditions, displays a gradual differentiation towards the osteogenic lineage with increasing density of dark granules that stained red with alizarin red dye.

5.3.1.2. Alizarin red extraction and quantification

Alizarin red dye was extracted after staining and quantified as described in detail in chapter 2 section 2.3.1. The amount of alizarin red dye extracted was normalized to DNA content of cells (section 2.3.2 and 3.3.1.2). Figure 5.3 and 5.4 shows digital images of the cells in a well

of six-well plate after staining with alizarin red dye. Cells grown in medium containing osteogenic supplements showed a significant increase in amount of alizarin red dye extracted in comparison to cells grown in medium devoid of them as shown in figure 5.5. Figure 5.6 is the DNA calibration curve used to normalize the amount of alizarin red dye. It needs to be highlighted here from data statistics point of view that all the measurements of alizarin red staining and quantification assays were made from two independent series of experiments done in triplicates due to same reasons discussed in Chapter 3 section 3.3.2.

MSCs grown in medium with and without Osoteogenic Supplements

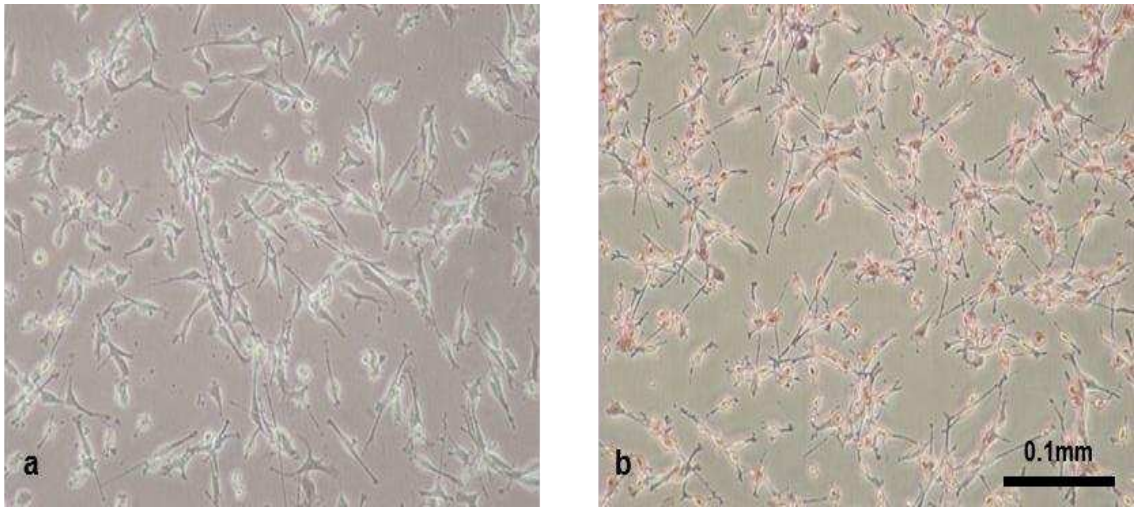


Figure 5.1: MSCs at day-0 prior to starting differentiation treatment (a) and stained with alizarin red dye (b). (All pictures were taken at 10X magnification).

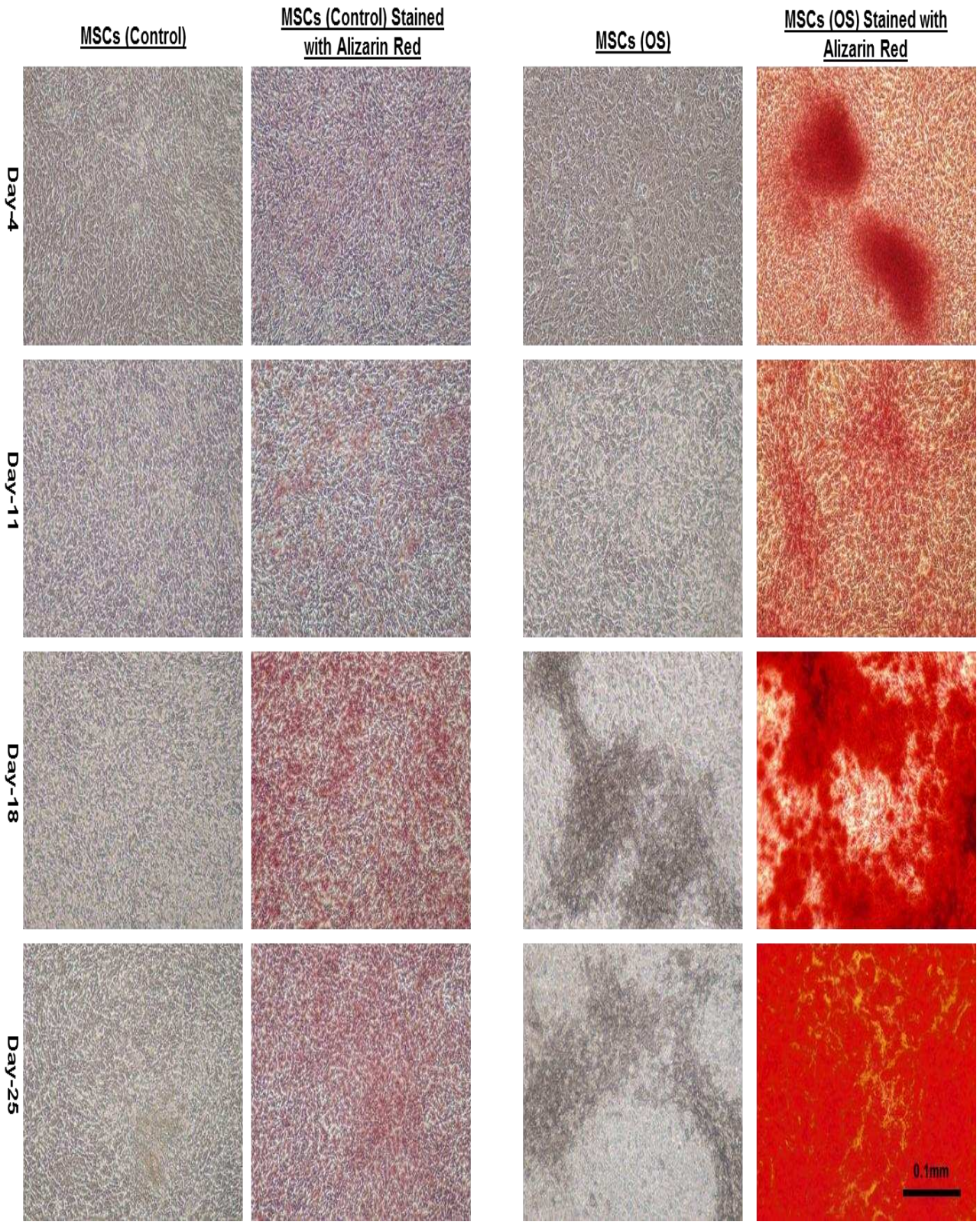


Figure 5.2: MSCs grown in medium containing and lacking osteogenic supplements for 4 weeks and then stained with Alizarin red dye. Dark areas surrounding cells are visible and stained red with dye (All pictures were taken at 10X magnification). It has been already detailed in section 3.3.1.2 in Chapter 3 that alizarin red stain in its own could not be taken as a measure of mineral deposition and so further data supplementing these observation is presented in section 5.3.1.3 and 5.3.2.

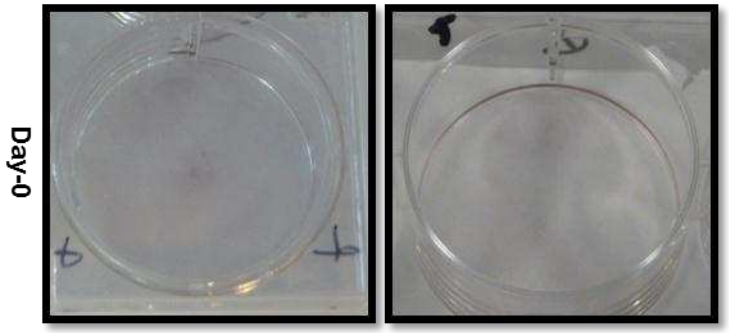


Figure 5.3: MSCs at day-0 prior to start of differentiation treatment and stained with alizarin red dye in six-well plates. No nonspecific staining was observed.

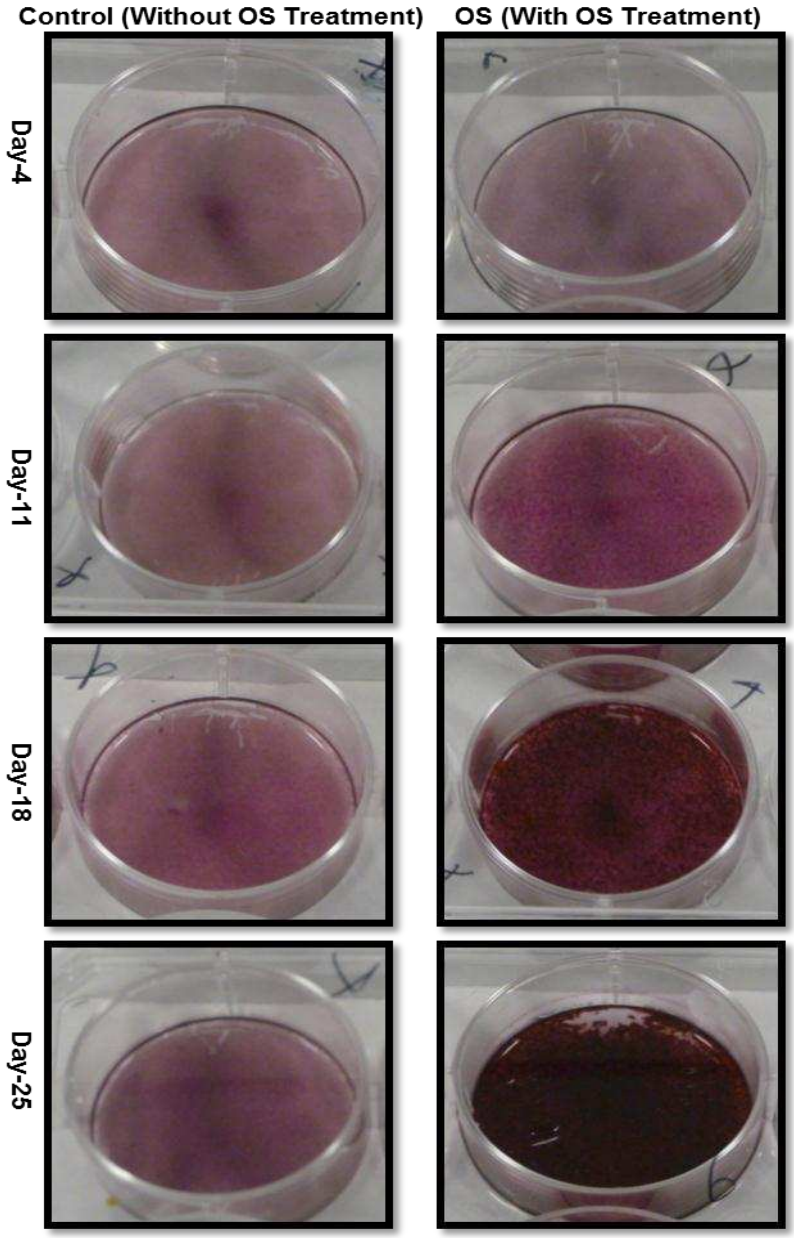


Figure 5.4: MSCs subjected to osteogenic differentiation for four weeks and stained with alizarin red dye for mineral deposition in six-well plates at various time points. Significantly intense staining is apparent in treated cells after two weeks compared to non-treated cell population.

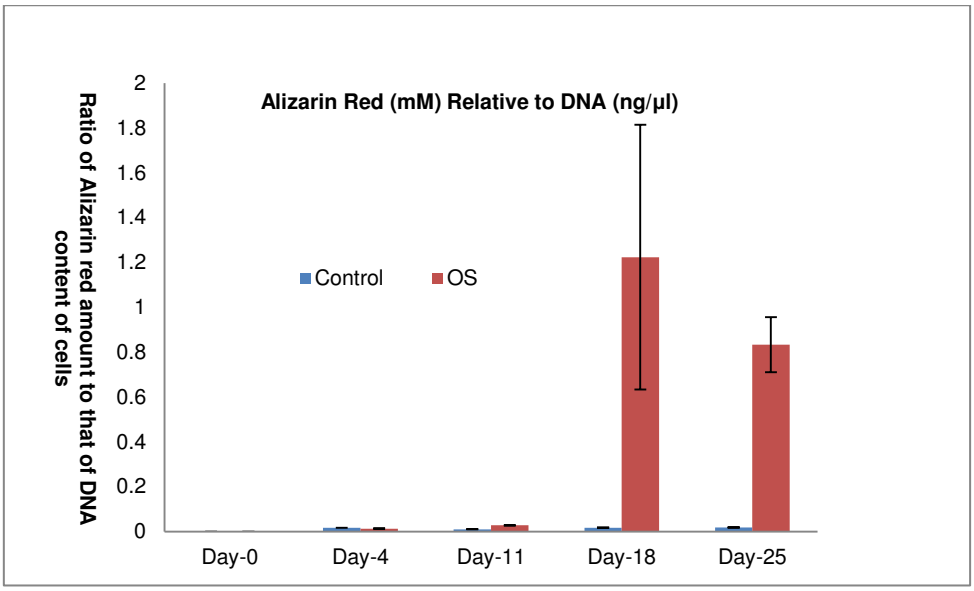


Figure 5.5: Mineral deposition was detected by alizarin red dye and then dye was acid extracted and quantified using a colourimetric assay. Data are collected from two plates, each analysed in triplicates and expressed as mean±SEM (n=2). There appeared to be a significant difference in amount of mineral deposition in treated and untreated cells.

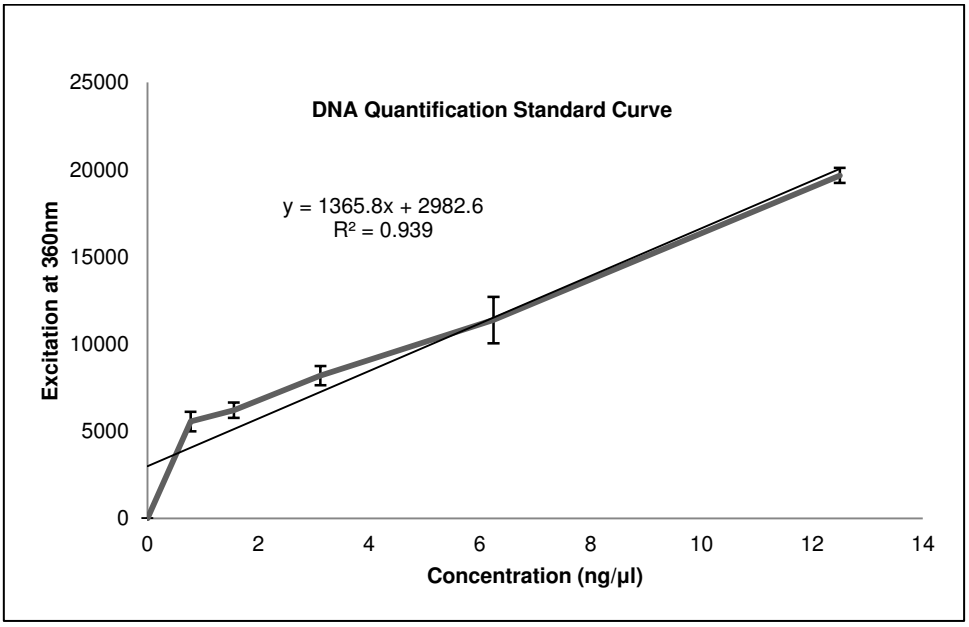


Figure 5.6: DNA calibration curve generated to normalize the amount of mineral deposition relative to cell contents of plates (data is collected from two plates and each analysed in triplicates so mean±SEM with n=2)

5.3.1.3. Immunofluorescence

Immunofluorescence was performed using antibodies against Sox2, Sox9, Osterix and Osteoclacin to detect the localization and expression of these marker proteins. Almost all of the cells in undifferentiated MSC culture were found to express Sox2 protein (Figure 5.10). Then only some of the cells were found to express Sox2 protein at day-4 in control cells and after wards no signal was detected (Figure 5.10). Cells grown in medium containing OS supplements did not show expression at any time point analysed beyond day-0 as shown in Figure 5.11.

Sox9 was detected in MSCs at day-0 but not all of the cells were observed to be expressive. Cells grown in medium lacking OS supplements showed a low level of protein expression in nuclei at day-4, day-11 and very few cells at day-18. No signal was observed at day-25 (Figure 5.12). While cells grown in medium containing OS supplements appeared to show gradual increase in positive signal with less positive cells at day-4 and day-11 and more at day-18 and day-25 (Figure 5.13).

Osterix protein was not found to be expressed at any time points in cells grown with and without OS promoting factors (Figure 5.14 and 5.15).

A strong cytosolic signal was observed for Osteocaclin starting from day-0 in undifferentiated MSCs and cells at later points i.e. day-18 and day-25 showed a high level of expression in both control (Figure 5.16) and OS populations (Figure 5.17). There was marked difference in level of expression in cells grown in OS medium.

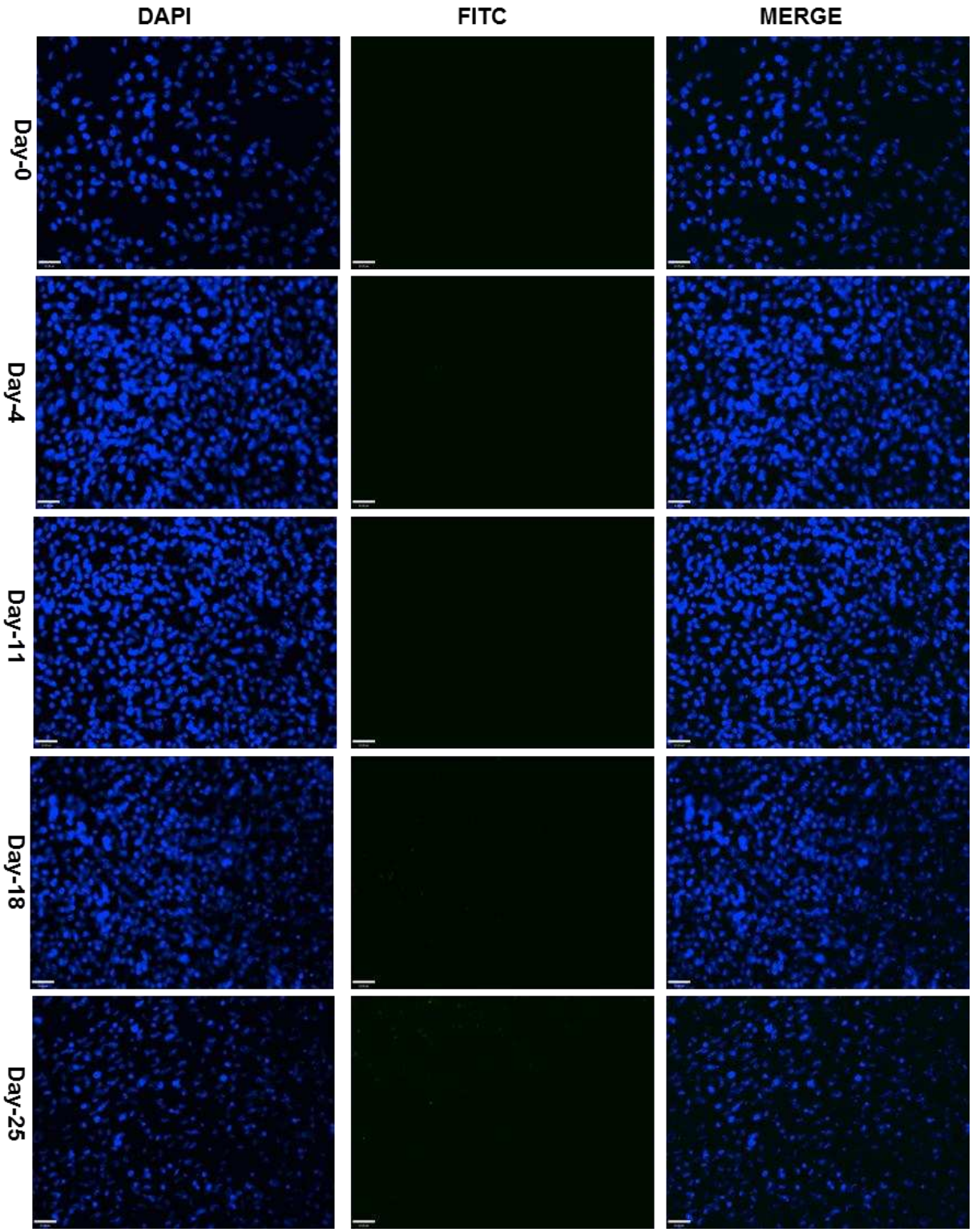


Figure 5.7: Mouse MSC (D1) cells grown in control medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

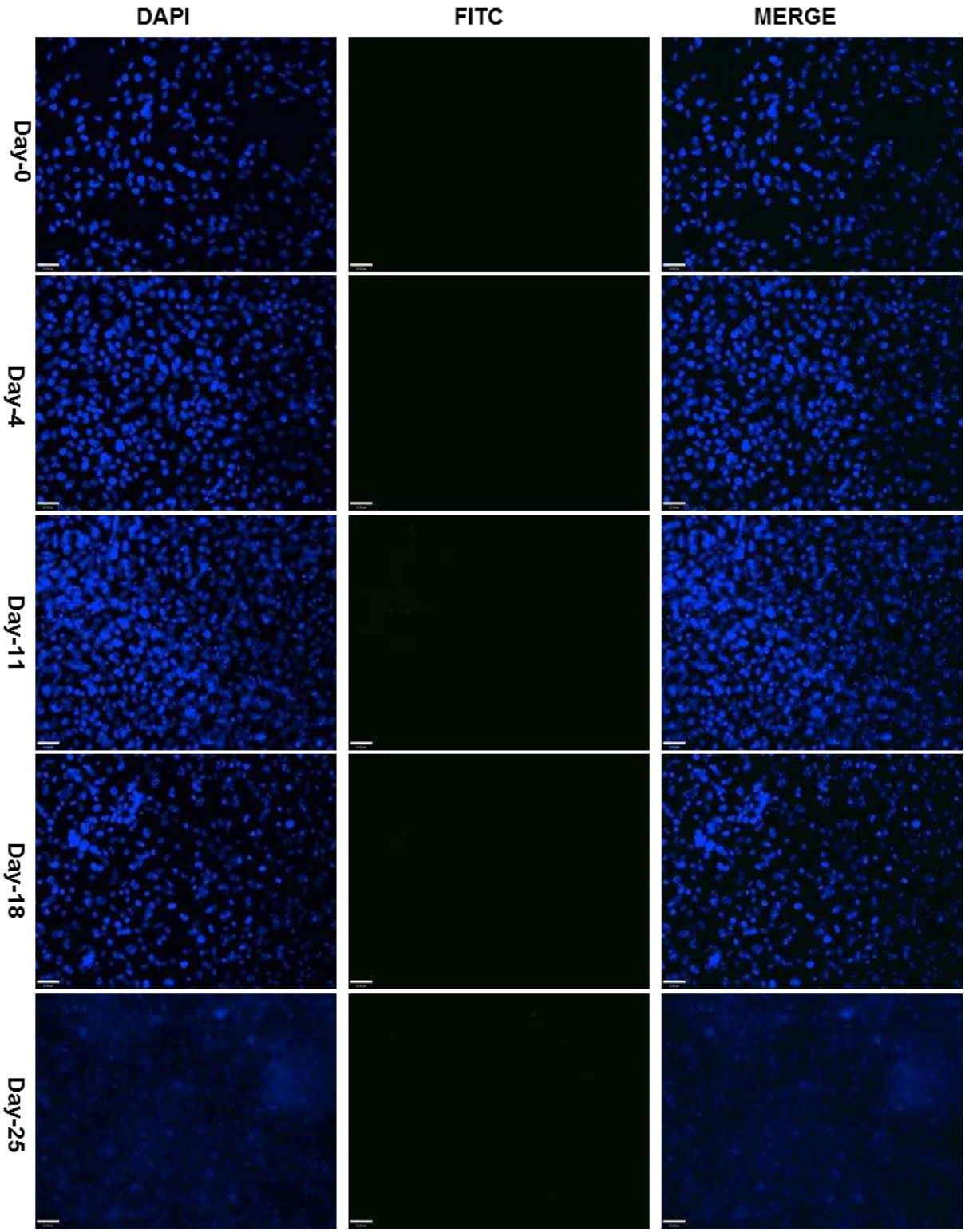


Figure 5.8: Mouse MSC (D1) cells grown in OS medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

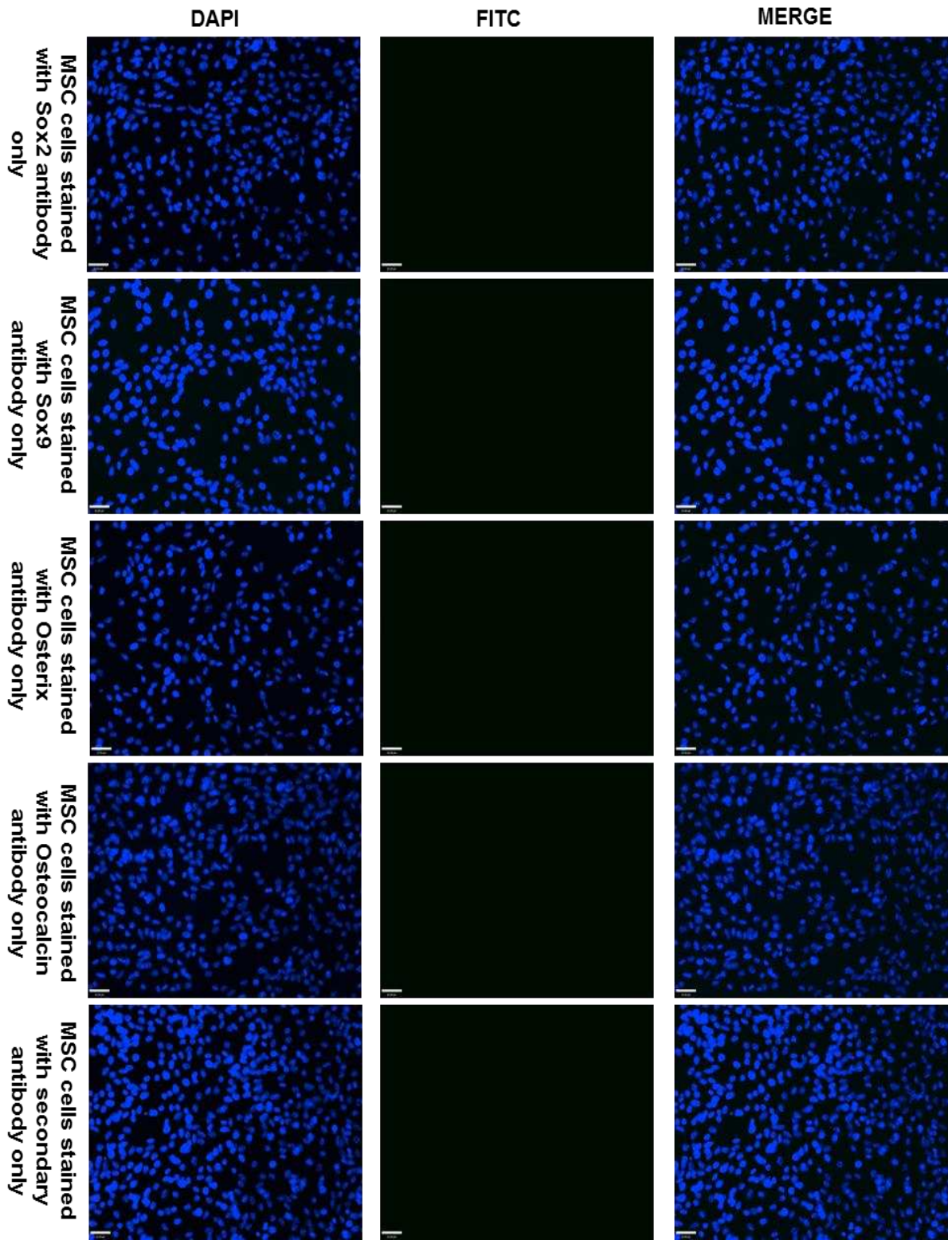


Figure 5.9: Mouse MSC (D1) cells stained without primary antibodies only and secondary antibody only to account for nonspecific fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

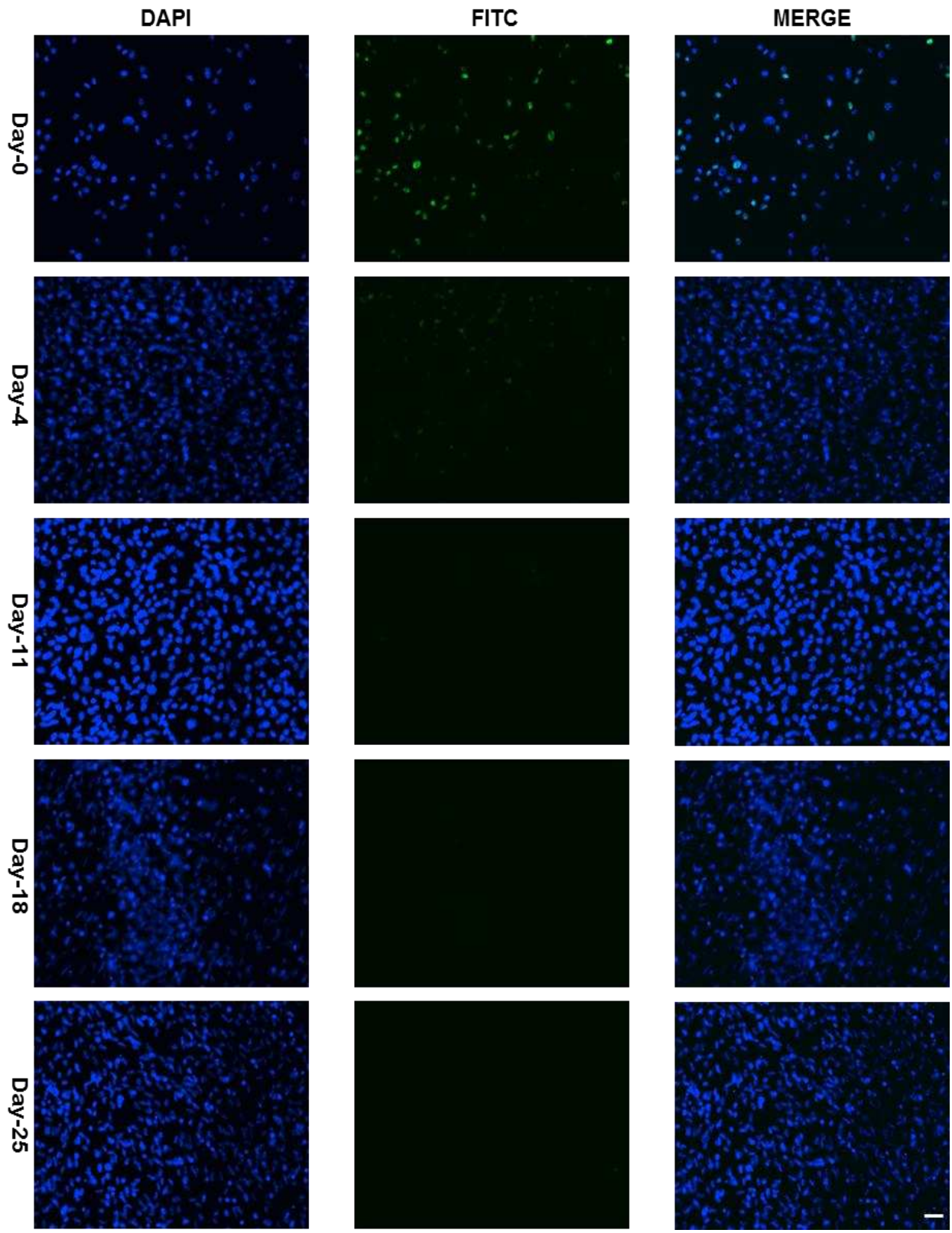


Figure 5.10: MSCs grown in medium lacking oesteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2) and found to be expressive at day-0 with some expression at day-4. No expression was detected after that. (All pictures were taken at 20X magnification and scale bar is 32µm).

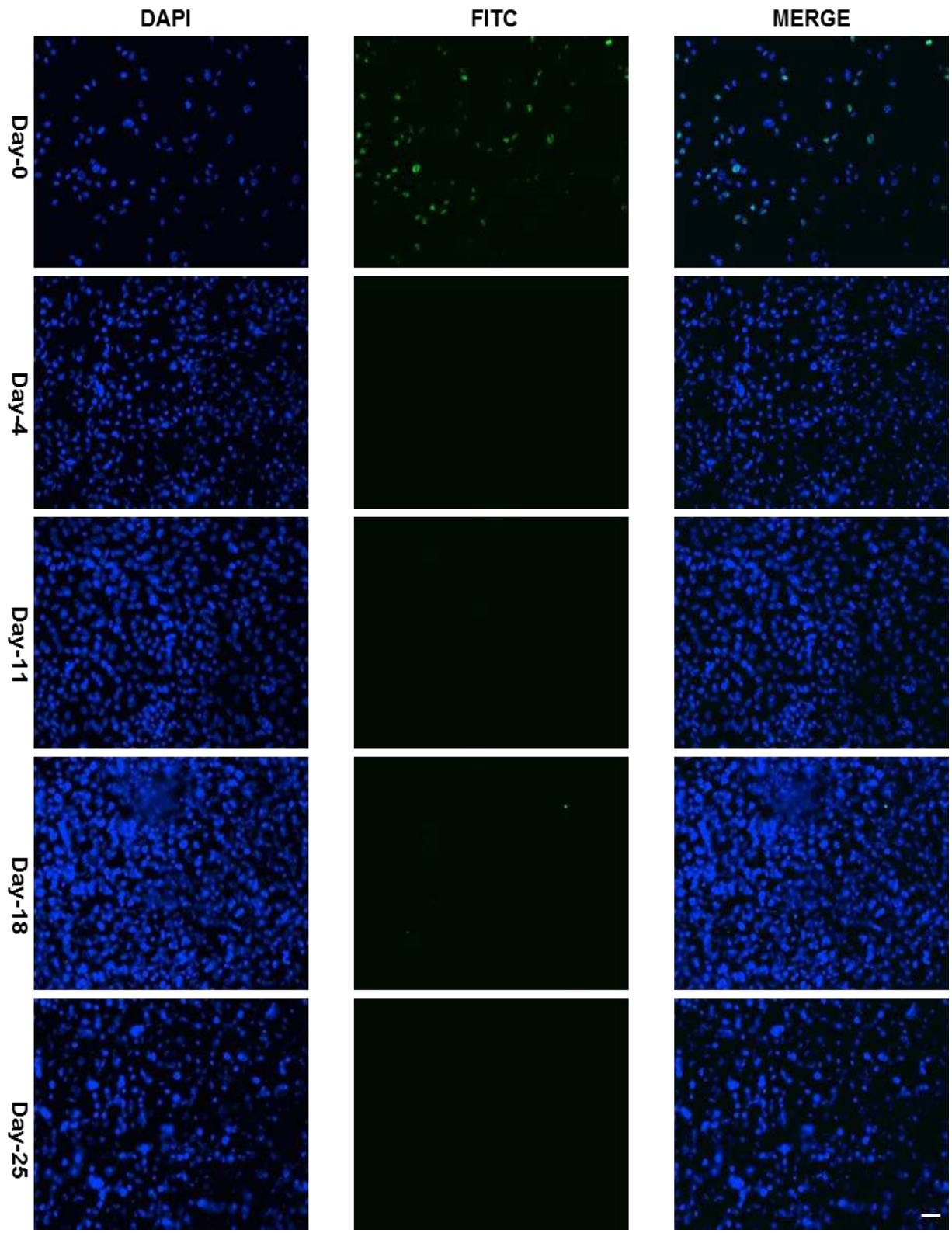


Figure 5.11: MSCs grown in medium containing oesteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2). Sox2 was found to be expressed by cells at day-0. No expression was detected beyond day-0 (All pictures were taken at 20X magnification and scale bar is 32µm).

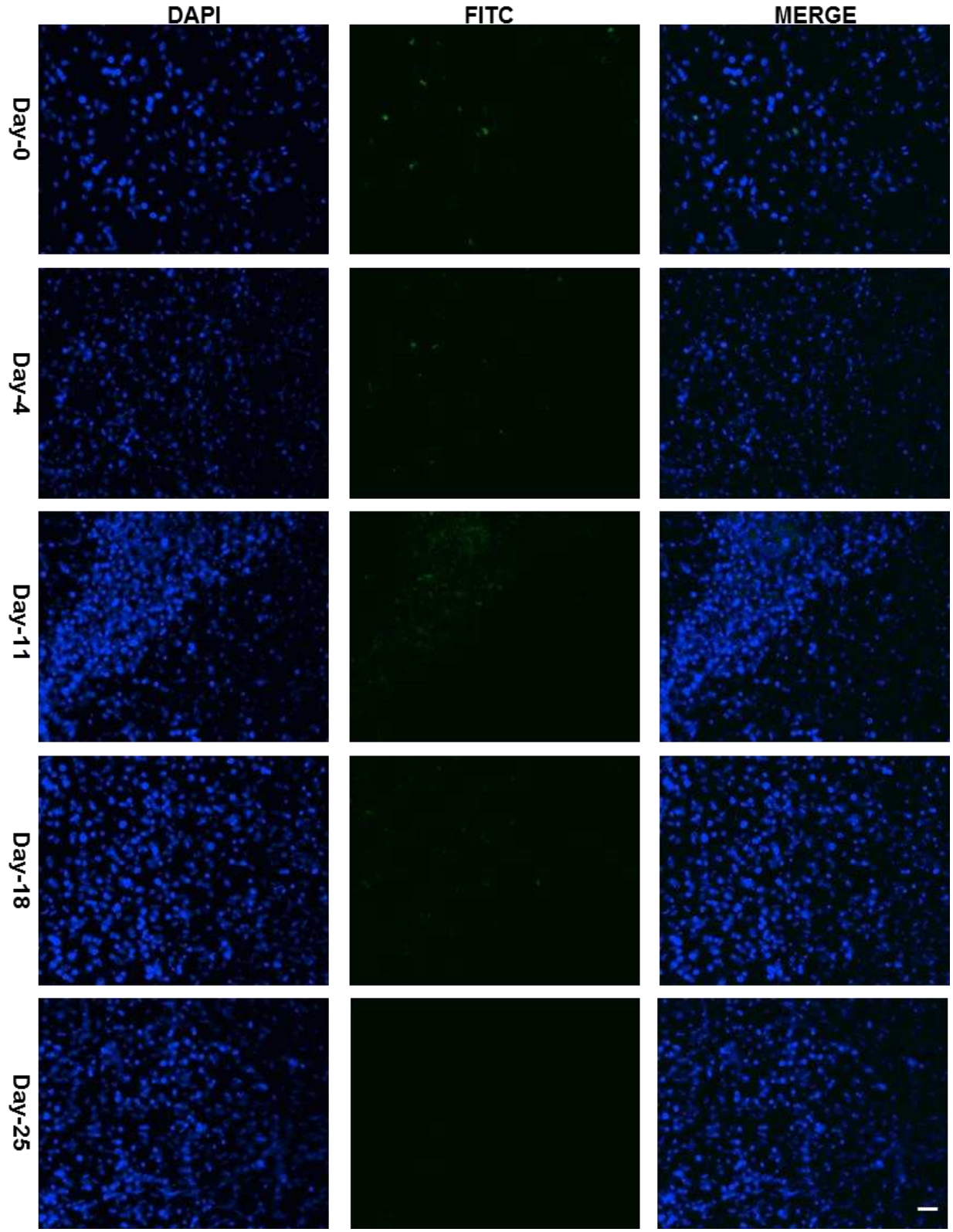


Figure 5.12: MSCs grown in medium lacking oesteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Sox9 was found to be expressed by cells at day-0. Few cells were found to be positive for Sox9 at day-4, more at day-11 than day-18 and no expression at day-25 (All pictures were taken at 20X magnification and scale bar is 32µm).

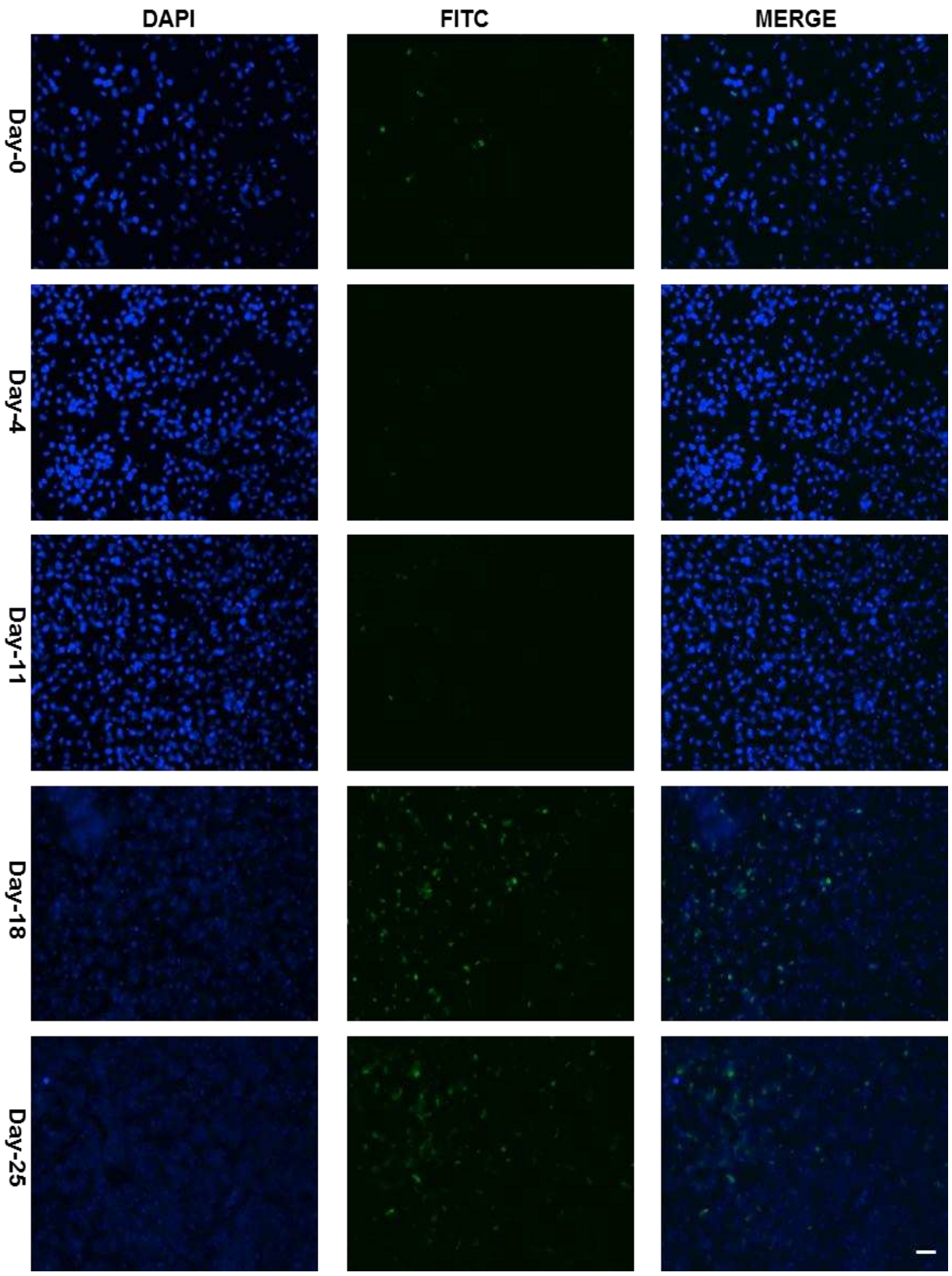


Figure 5.13: MSCs grown in medium containing oesteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Sox9 was found to be expressed by cells at day-0. Few cells were found to be positive for Sox9 at day-4 and then gradual increase at day-11, day-18 and day-25 (All pictures were taken at 20X magnification and scale bar is 32µm).

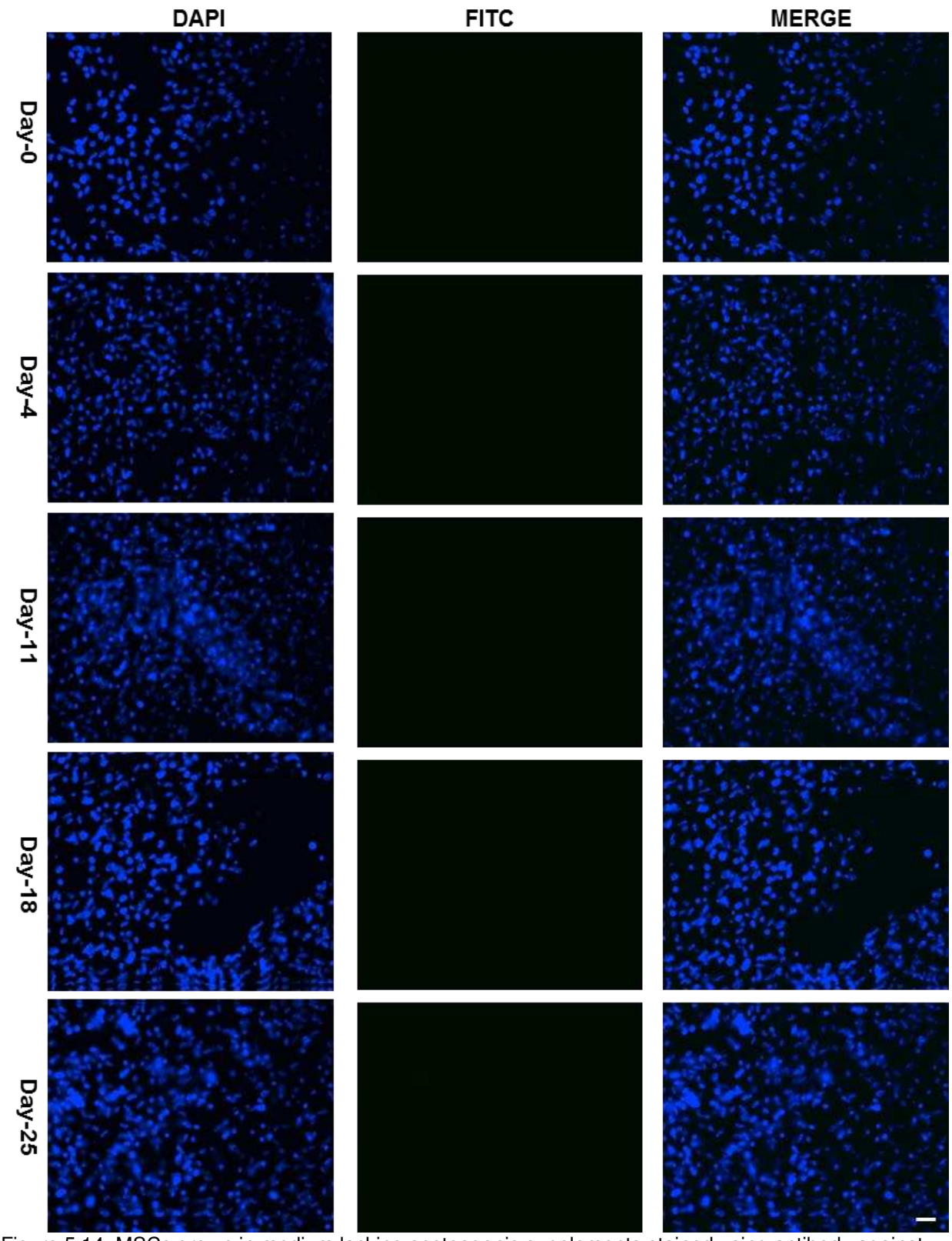


Figure 5.14: MSCs grown in medium lacking oesteogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No signal was detected at any time point analysed (All pictures were taken at 20X magnification and scale bar is 32µm).

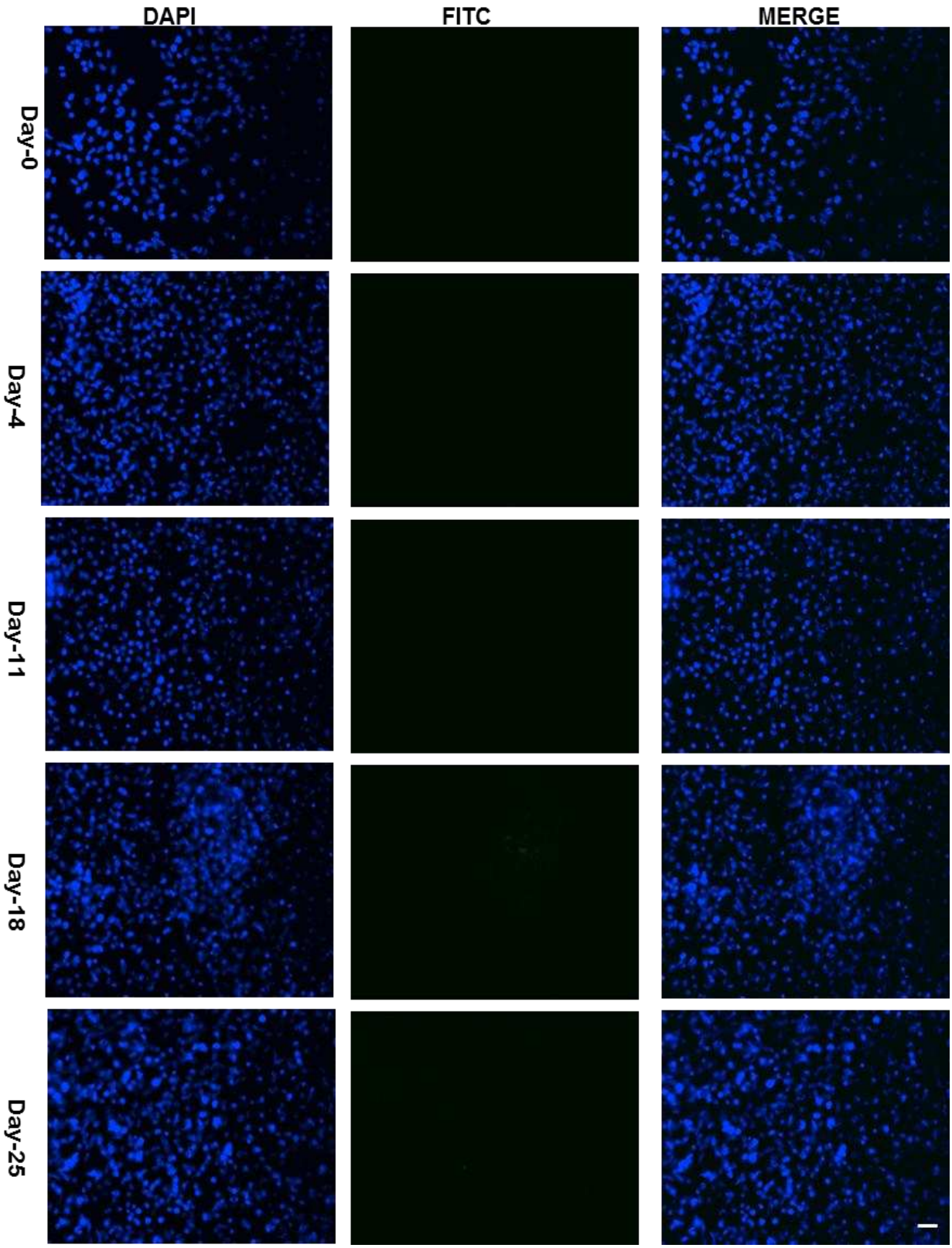


Figure 5.15: MSCs grown in medium containing oesteogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No positive signal was observed at day-0, 4, 11, 18 and 25 (All pictures were taken at 20X magnification and scale bar is 32µm).

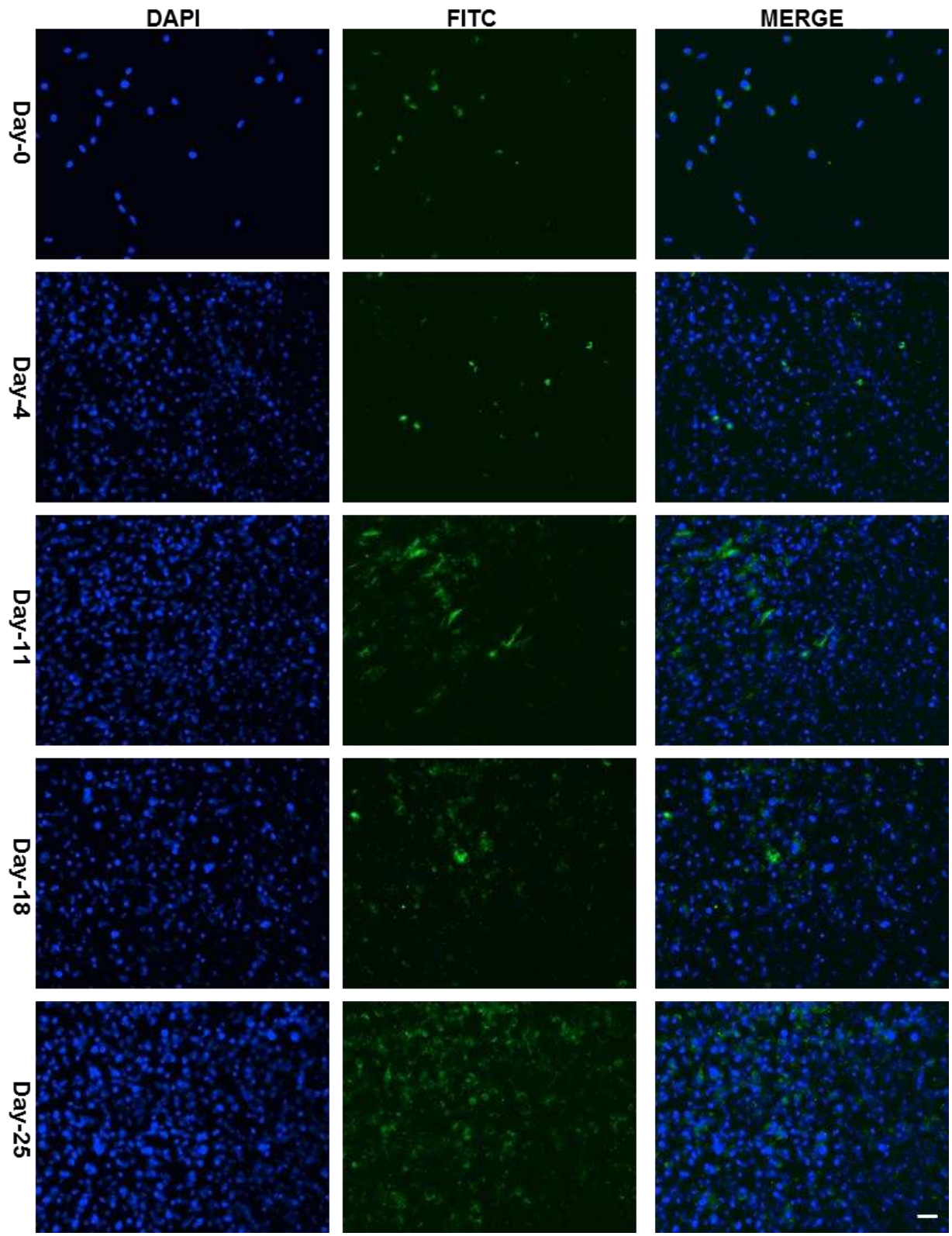


Figure 5.16: MSCs grown in medium lacking oesteogenic supplements stained using antibody against Osteoclacin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osteocalcin). Cell found to be positive starting at day-0 with gradual increase in intensity of signal at day-4, 11, 18 and 25 (All pictures were taken at 20X magnification and scale bar is 32µm).

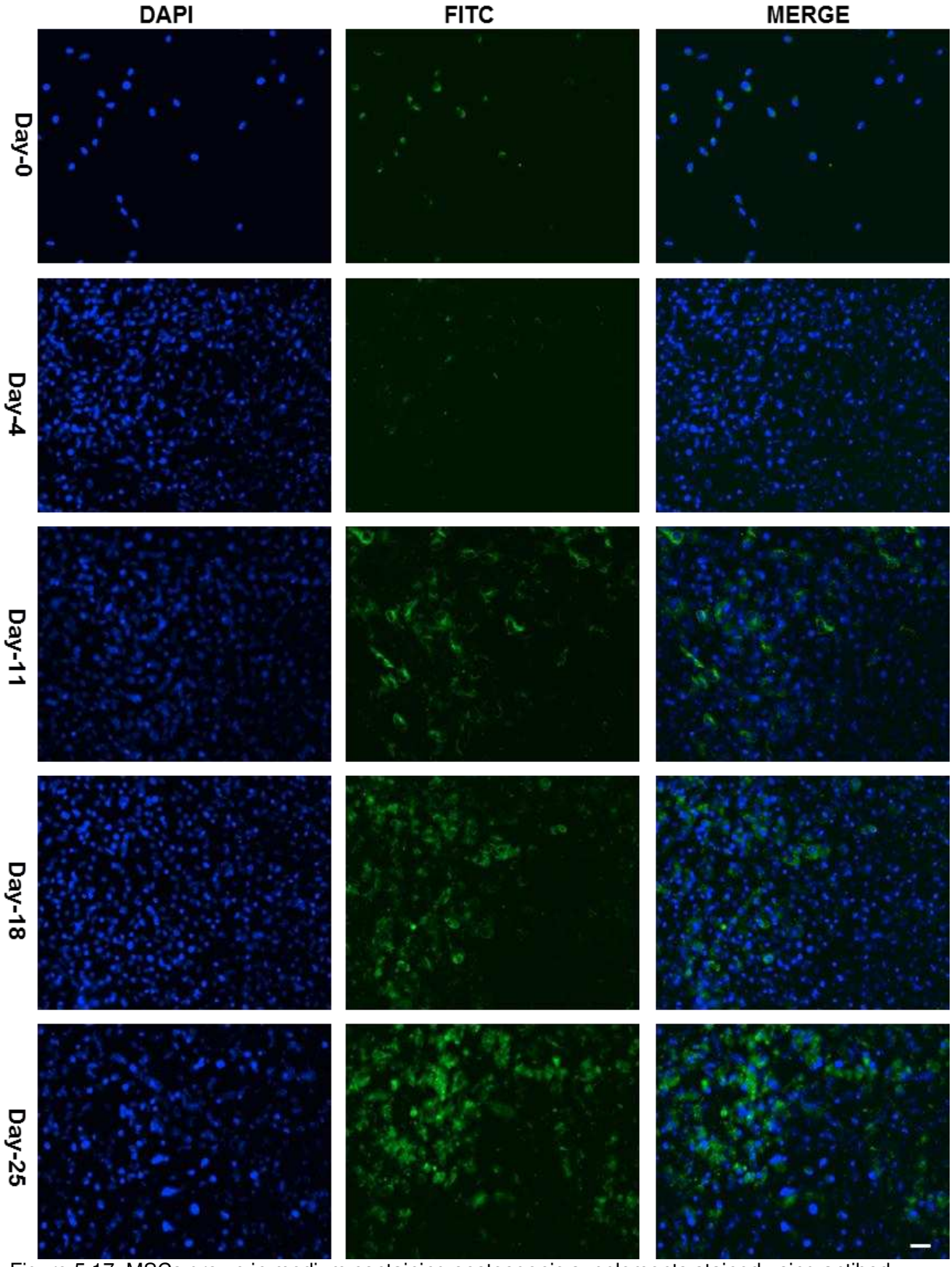


Figure 5.17: MSCs grown in medium containing oesteogenic supplements stained using antibody against Osteoclacin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osteocalcin). A positive singal was first seen at day-0 with increased with time along the differentiation time course (All pictures were taken at 20X magnification and scale bar is 32µm).

5.3.2. Molecular Analysis

5.3.2.1. Gene Expression Analysis by reverse transcription PCR (RT-PCR)

Gene expression studies with GAPDH as internal control and Sox9, Alkaline phosphatase, Runx2, Osteirx, Osteopontin, and Osteocalcin as osteo-specific markers were carried out on random primed cDNA synthesized from RNA, extracted from cells at different time-points during differentiation (section 2.2.3). Figure 5.18 displays representative results obtained from these analyses.

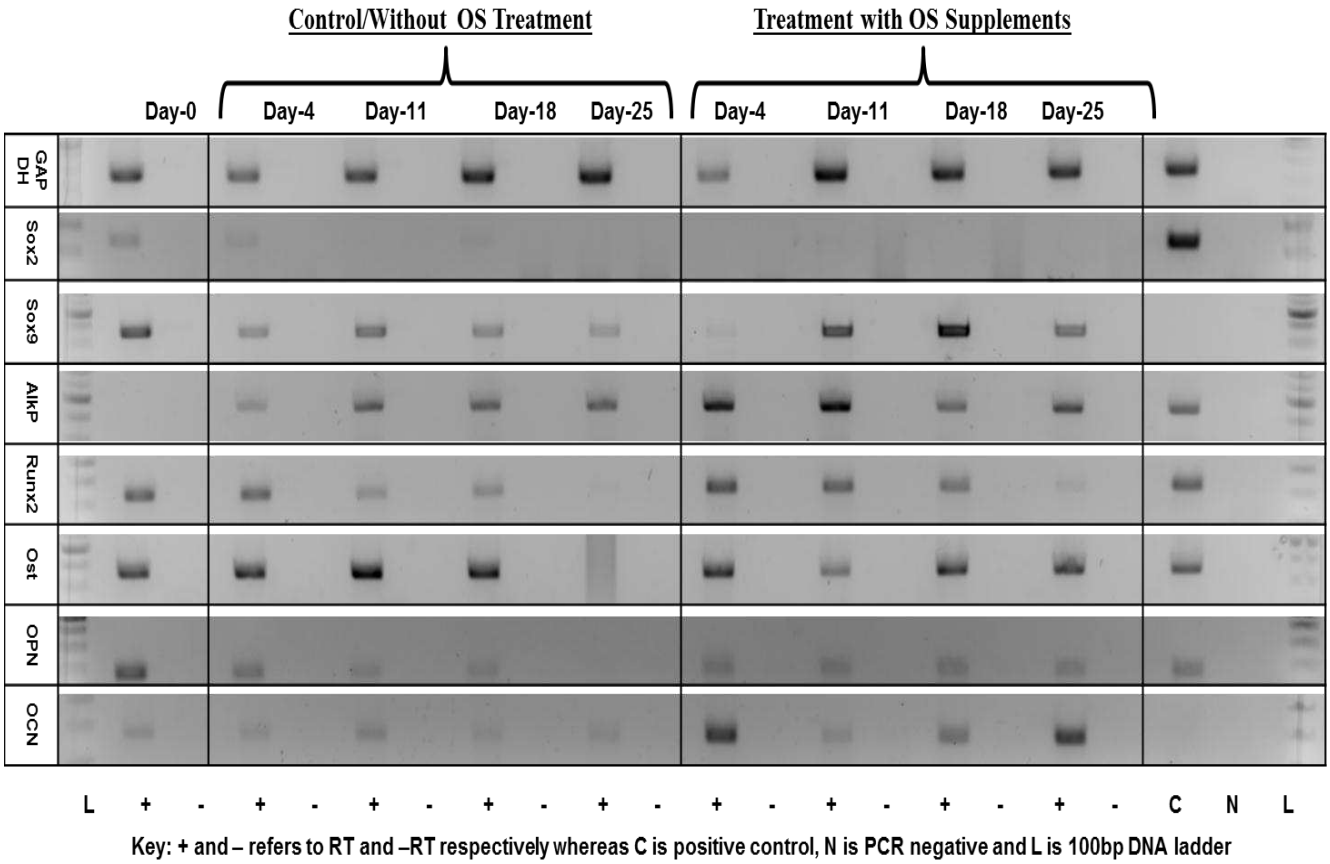


Figure 5.18: Gene expression analysis using random primed cDNAs of MSC osteogenic time course. GAPDH was used as internal control. These are the results from experiments done in triplicates from three independent biological replicates (n=3).

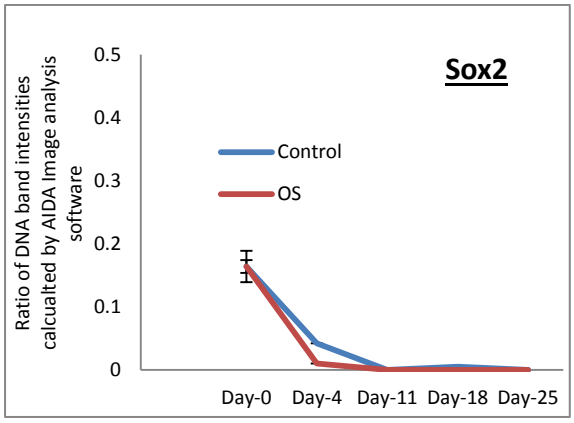


Figure 5.19: Semi-quantitative gene expression analysis showing expression of Sox2 in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean±SEM (n=3).

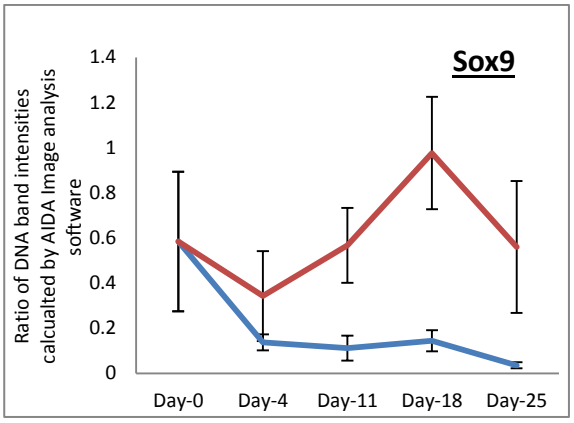


Figure 5.20: Semi-quantitative gene expression analysis showing expression of Sox9 in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean±SEM (n=3).

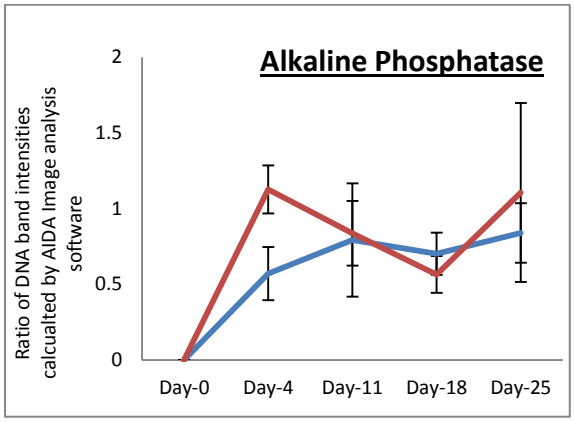


Figure 5.21: Semi-quantitative gene expression analysis showing expression of Alkaline Phosphatase in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean±SEM (n=3).

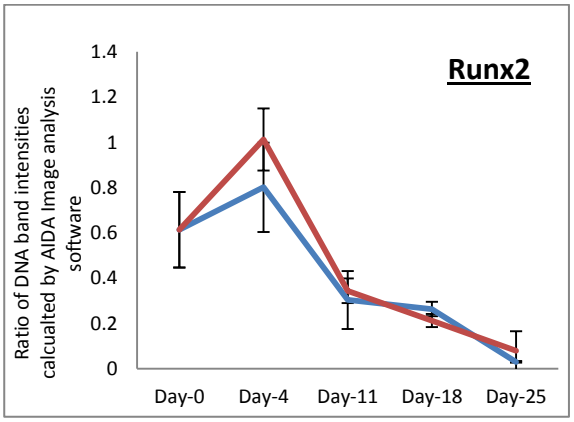


Figure 5.22: Semi-quantitative gene expression analysis showing expression of Runx2 in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean±SEM (n=3).

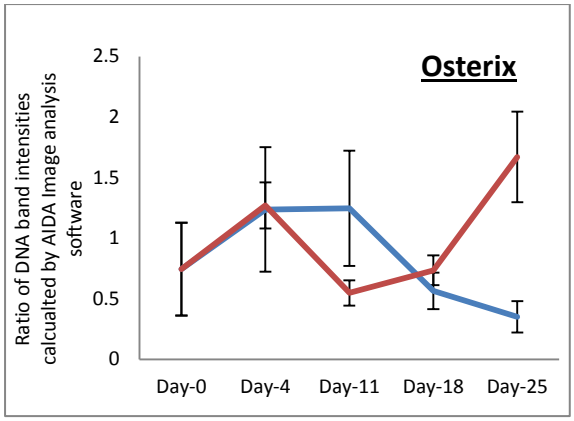


Figure 5.23: Semi-quantitative gene expression analysis showing expression of Osterix in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean±SEM (n=3).

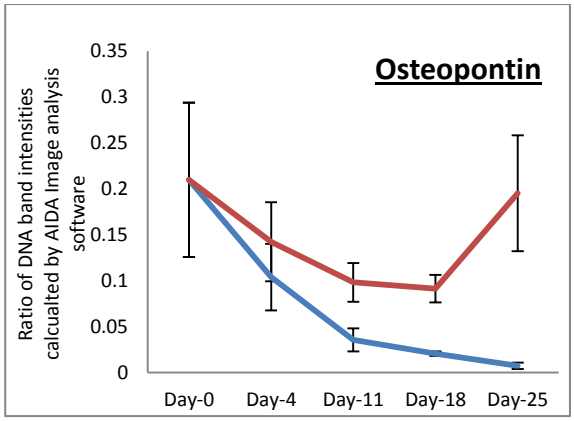


Figure 5.24: Semi-quantitative gene expression analysis showing expression of Osteopontin in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean±SEM (n=3).

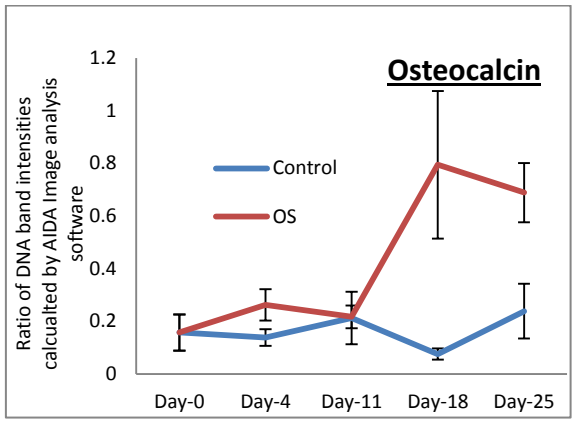


Figure 5.25: Semi-quantitative gene expression analysis showing expression of Osteoclacin in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean±SEM (n=3).

Sox2 was found to be expressed at day-0 and then a very low level of expression was observed at day-4 in control cells which correlates well with the protein expression as observed by immunofluorescence (Figure 5.10). While cells grown in medium containing OS supplements did not show Sox2 expression at RNA level (Figure 5.18 and 5.19) and protein level (Figure 5.11) after day-0.

Sox9 seemed to be showing differential gene expression where expression appears to be stronger at day-18 and day-25 in OS cells in comparison to cells grown in control medium (Figure 5.18 and 5.20). The same was observed with Sox9 expression at protein level by immunofluorescence (Figure 5.12 and 5.13).

Alkaline phosphatase appeared to be upregulated at day-4 in OS cells and then going down at day-18. Cells grown in control medium showed similar pattern of expression but less than OS treated cells (Figure 5.18 and 5.21). Similarly Runx2 found to be down regulating after day-4 along differentiation time course in both treated and non-treated cell populations (Figure 5.18 and 5.22).

Osterix, Osteopontin and Osteocalcin were found to be expressed at all time-points examined where Osteoclain seems to be expressed in higher levels at later points of differentiation i.e. day-25 (Figure 5.18, 5.23-5.25). Osteocaclin protein was also found to be expressed at all time-points with stronger signal at day-18 and day-25 (Figure 5.16 and 5.17).

5.3.2.2. Analysis of DNA methylation at SRR1 and SRR2 by MS-PCR

In order to look at DNA methylation changes occurring at SRR1 and SRR2, MS-PCR was carried out in the similar way as already detailed in chapter 2 section 2.3.2.4 and chapter 3 section 3.2.3.2. Both SRR1 and SRR2 regions of Sox2 were observed to be methylated at all time-points i.e. day-0, 4, 11, 18 and 25 in both undifferentiated and differentiated cells grown in osteogenic differentiation medium (Figure 5.28-5.29).

Figure 5.26 shows the result of PCR carried out on DNA prepared from MSCs grown in control and OS medium at different time points and then digested with MspI and HpaII enzymes. A region of DNA containing Msp sites but reported to be unmethylated was used as control (section 3.2.3)

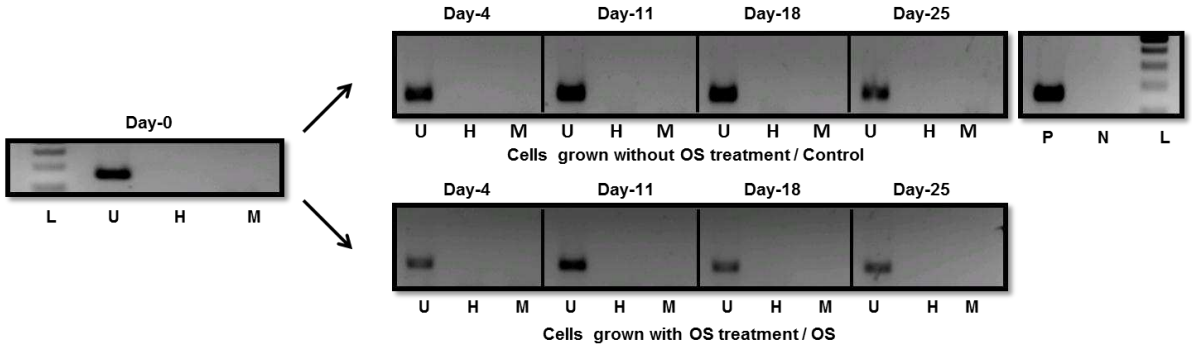


Figure 5.26: mAprt promoter region used as control and showing that no amplified product was observed in *Msp*I and *Hpa*II digested samples so digestion is not partial.

Figure 5.27 is the result of PCR carried out on same set of samples as described above. This is the genomic region known to be methylated and contains Msp sites.

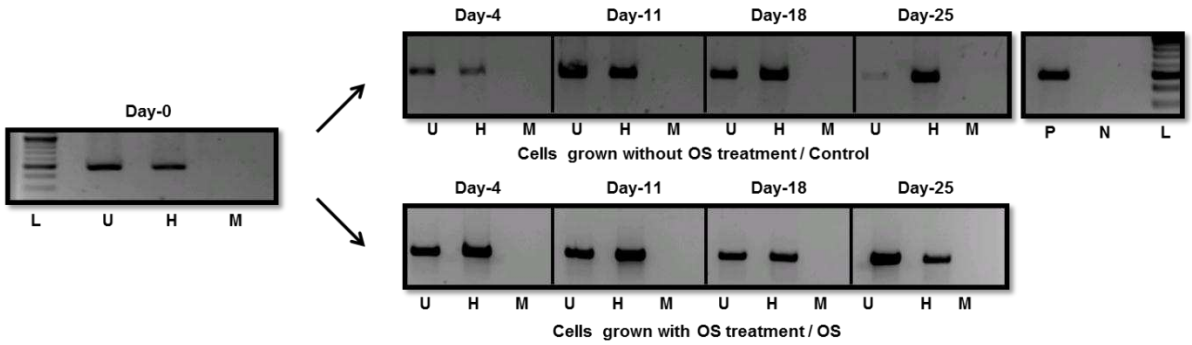


Figure 5.27: mIgf2R-DMR2 region used as control and showing that no amplified product was observed in *Msp*I digested samples and only *Hpa*II digested fragments amplified.

SRR1 and SRR2 regions of Sox2 were found to be methylated at time points selected for analysis in both control and OS treated cell populations (Figure 5.28 and 5.29). This technique only allows to study DNA methylation at any genomic region that contains Msp sites and when all of them are methylated. And if any of these in unmethylated or partially methylated then due to digestion of DNA amplified product won't be seen.

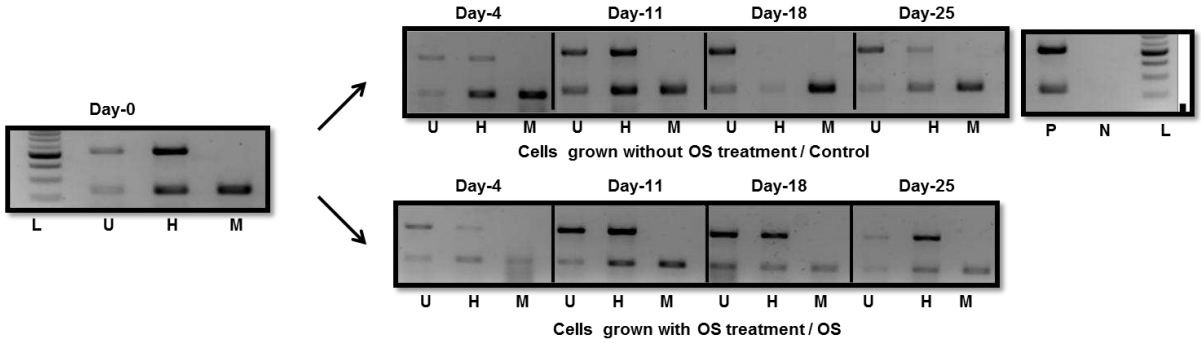


Figure 5.28: SRR1 region of Sox2 was found to be methylated at every time point examined by MS-PCR

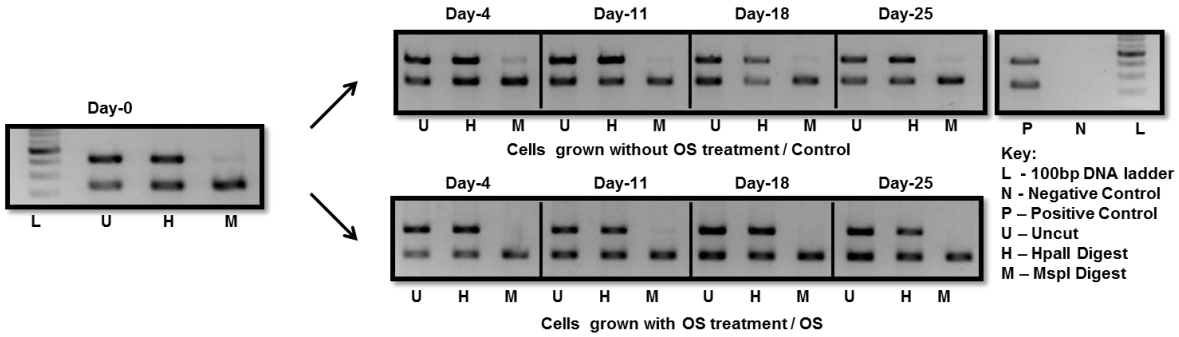


Figure 5.29: SRR2 region of Sox2 was found to be methylated at day-0, 4, 11, 18 and 25 in both control and OS cells by MS-PCR

5.4. Discussion

Bone development in vitro has been traditionally analysed by the mineralized bone nodules formation (Heng et al., 2008). As is apparent is Figure 5.2, mineralization as black layer around the cells under the microscope can be clearly seen at day-11 of differentiation showing a gradual increase by day-25. Further staining with alizarin red dye showed the darkening of deposited granular matrix while control cells did not show such a strong staining. This dye was subsequently extracted and quantified using an already described assay to assess the extent of mineralization (Gregory et al., 2004). Figure 5.5 shows very clearly that treated population of MSCs have produced more stain than non-treated cells and the difference is really apparent after two weeks of culture. As detailed in the discussion section of the chapter 3 (section 3.4), medium used for oseteogenic fate induction could lead to non-specific mineralization. Therefore, further characterization of differentiating MSCs cells was carried out using cell surface and other molecular markers by immunocytochemistry and gene expression profiling.

As described previously ostetogenesis is a complex process and the expression pattern of various markers associated with osteoblasts is organized temporally and sequentially (Zur Nieden et al., 2003). Type I collagen, Alk-P and osteonectin have been reported to appear at the end of the proliferation phase; osteopontin followed by bone sialoprotein and Runx2 at the start of mineralization phase; and finally osteoclacin at high level during mineralization. All these markers can have varying times of expression onset in culture dependent on cell line and methods of differentiation (Duplomab et al., 2007).

Sox2 has been reported to maintain self-renewing osteoprogenitor cell population and favours their maintenance over differentiation into mature osteoblasts (Roy et al., 2010). It has also been reported that cells impaired in osteogenic differentiation show high level of Sox2 expression (Muraglia et al., 2008). Moreover, Sox2 has been reported to inhibit in vitro murine osteoblastic differentiation by inhibiting Wnt signalling (Mansukhain et al., 2005). Here Sox2 was observed both at RNA and protein level at day-0 in undifferentiated cells and then very small expression was detected at day-4 in control cells. Cells grown in OS medium did not show Sox2 expression neither at RNA level nor at protein level hinting at the possibility that cells could have become committed and are no longer in proliferative phase.

Sox9 is known to be expressed in mesenchymal condensation during the deposition of cartilage and plays a central role in skeletogenesis (Wright et al., 1995). Based on the location of MSCs, they differentiate to form chondrocytes or osteoblasts and Sox9 is one of the important determining factor (Fujii et al., 2005). Here in these experiments Sox9 was found to be down regulated both at RNA and protein level after two weeks of MSC culture in differentiation medium compared to control cells. Cells maintained a lower level of RNA and protein expression at all time-points analysed except day-25 in control where no protein was detected.

Runx2 and Osterix are two central transcription factors in determining the commitment of osteoprogenitors cells to osteoblasts where Osterix is thought to act downstream of Runx2 (Komori et al., 2006). Runx2 was found to be upregulated at day-4 in OS cells and then down regulated by day-25. A significant expression of Osterix was detected starting from day-0 and at all time-points during differentiation while no protein expression was detected at any time point. A previous study has shown that Osteirx expression stimulates mesenchymal stem cell

proliferation in a partially committed stage of osteoblast precursor (Kim et al., 2005). Similarly alkaline phosphatase, osteopontin and osteoclacin which are reported to be expressed in higher amounts in osteoblasts as detailed in chapter 3 (section 3.4), were observed to be present at all time-points analysed in both control and OS treated population.

Together these observations suggest that this MSC population seems to be already enriched in expression of markers associated with late stage of osteoblasts and at the same time expressing marker associated with early stages of osteoblast commitment at later stages i.e. in third and fourth week of culture. This could be due to the heterogeneity in starting population that some of the cells have already become committed to osteoprogenitors and hence are preosteogenic. There is also the possibility of asynchronous differentiation i.e. some cells have yet to start mineralizing so expressing early stage markers while others have mineralized.

DNA methylation has been reported as a major mechanism of gene silencing (discussed in chapter 3 section 3.4). Two regulatory regions of Sox2 namely SRR1 and SRR2 were chosen for analysis (section 1.5 and 1.6) and found to be constitutively methylated at all time-points when analysed with MS-PCR. Given the limitation of the technique to resolve DNA methylation status at each CpG site, bisulphite sequencing was attempted. But unfortunately none of the region could be PCR amplified after bisulphite treatment which could then be analysed by DNA sequencing. Hence further work is needed to optimize the reaction conditions for PCR amplification.

Adult stem cells have been now given focus regarding neoplastic transformation so that they could be exploited for their differentiation potential while keeping the self-renewing capacity

(Serakinci et al., 2004). Further work investigating the mechanisms regulating differentiation in this transformed cell line would be of interest to gain a molecular insight into induction of proosteogenic potential as is apparent by these observations.

Chapter 6 – Discussion

6. Discussion

Development is a process resulting in sequential reduction of differentiation potential of pluripotent progenitors involving activation or repression of various gene networks as cells acquire more specialized characteristics. This differentiation into specialized tissues and organs proceeds without any changes in DNA sequences of genomes of the cells so can be regarded as under epigenetic control (Reik, 2007). Indeed it has been experimentally shown that genomes from differentiated cells of animals can be used to clone animals proving that cells do not lose their genetic material during differentiation; rather regulated and controlled gene expression determine and maintains cell fate (Gurdon et al., 1975; Wilmut et al., 1997).

Regulation at the transcriptional level is central to the process because both spatial and temporal expression of transcribed proteins determines the identity of a cell, tissue or organ (Prior and Walter, 1996). An important component of transcriptional regulation is transcription factors which bind to DNA and co-ordinate the cellular decision making process, making it irreversible and free of errors which could potentially lead to developmental abnormalities and disorders. Lately, such transcription regulators have been identified that maintain the differentiation potential of embryonic stem cells, and forced expression of these factors can even reprogram somatic genomes to an undifferentiated state (Takahashi et al., 2006; 2007; Park et al., 2008).

Epigenetic modifications such as DNA methylation of CpG dinucleotides, modification of histone tails mainly by acetylation and methylation, other non-histone proteins and regulatory RNAs affect and change gene expression patterns during cellular differentiation along with transcription factors (Jaenisch and Young, 2008). All of these factors acting together lead to

the generation of cell and tissue specific gene expression patterns reflected in their genotype under given circumstances and govern their phenotype (Morgan et al., 2005). So interplay between these factors at different stages of development regulates pluripotency of progenitors and their differentiation at the right time for example by maintaining reversible repressive expression of genes required at later stage of development at earlier stages and/or irreversible silencing of pluripotency associated genes after cellular commitment and differentiation (Reik, 2007).

Embryonic stem cells have been shown to be able to differentiate into all lineages derived from embryos in vitro and hence can be used as a model system not only to study developmental programming and reprograming events but also, once these events are known can be used to create differentiated cell types for transplantation therapies in clinical applications (Solter and Gearhart, 1999). Sox2 has been reported to be indispensable for mouse embryonic stem cells self-renewal and also, is one of the transcription factors used to reprogram somatic cells.

This study specifically looked at DNA methylation status of two evolutionary conserved regulatory regions of Sox2 namely SRR1 and SRR2 in undifferentiated mouse embryonic stem cells and after subjecting them to differentiation treatment leading to osteogenic and neural lineages that are derived from germinal ectoderm and mesoderm respectively. Mouse mesenchymal stem cells, the physiological precursor of osteoblasts were subjected to a similar differentiation treatment in parallel, so as to compare and contrast the changes in DNA methylation at these regions when same cell types are obtained from embryonic and adult origin stem cells. The objective was to examine the role of DNA methylation of regulatory regions of Sox2 in cell type specific expression pattern.

DNA sequences of short length often known as regulatory elements are basic components in gene regulation and can be found in promoter, enhancer, near the transcription start site (TSS) or far away from TSS and can be either cis or trans acting (Sikorski and Buratowski, 2009). The combinatorial action of a given set of regulatory elements for a particular gene would determine its expression in tissue specific and environmental specific manner (Latchman, 2004; Levine and Tjian, 2003). The Sox2 gene has been reported to contain two evolutionary conserved upstream and downstream (~4kb from transcription start sites) enhancers designated as Sox2 Regulatory Region 1 and 2 (SRR1 and SRR2) in mouse and humans (Tomioka et al., 2002; Sikorska et al., 2008).

SRR1 contains binding site for POU transcription factors while SRR2 binds Oct4-Sox2 and are essential for Sox2 expression both in ES cells and neural stem cells (Catena et al., 2004; Tomioka et al., 2002). It has been previously shown that these regulatory sequences control region specific expression of Sox2 in neural stem cells such that expression is seen in telencephalon but not spinal cord stem cells (Miyagi et al., 2006; Zappone et al., 2000). Another study has found that these enhancers exhibit differential DNA methylation and histone H3 acetylation during human neural progenitor's differentiation into astrocytes and neurons affecting expression level of Sox2 in these different cell types (Sikorska et al., 2008). Their study has found that methylation of SRR2 enhancer particularly at highly conserved site +4250, correlated with silencing of Sox2 expression in neurons while SRR1 methylation in both astrocyte and neurons linked to gene silencing and cell cycle exit. They concluded that differential DNA methylation of these two enhancers of Sox2 in neural precursors and terminally differentiated cells mediumte neurodevelopment and regulate gene expression transiently and permanently.

Given that these sequences are highly conserved in mouse, this study looked at differentiating neuronal cells from embryonic stem cells and three individual CpGs analysed in core enhancer region of SRR2 were found to be unmethylated in both undifferentiated ES cells and differentiated cells. At the level of this analysis, it could not be concluded that all of the CpG sites remained unmethylated during differentiation. It is also possible that differentiated cells contained a smaller proportion of terminally differentiated neurons and presence of other cell types as cells were not sorted prior to analysis might, have potentially masked the low level of methylation (chapter 4).

Both SRR1 and SRR2 appeared to be methylated in ES cells grown in medium promoting differentiation towards osteoblasts when analysed by MS-PCR except at day-0 (undifferentiated state). As described above, these two enhancers contribute towards ES specific expression of Sox2 so could be unmethylated in undifferentiated cells. This could also be due to the fact that major epigenetic reprogramming events take place upon cell differentiation and commitment (Reik et al., 2001). So these regions might be unmethylated at all of CpG sites in undifferentiated ES cells and upon differentiation at the day-3 embryo bodies stage start setting up some methylation marks.

MS-PCR analysis described in Chapter 2 section 2.3.3 showed that both SRR1 and SRR2 became methylated at day-3 and then appeared to be methylated at all time-points analysed thereafter. No Sox2 expression was detected at the RNA level after seven days of treatment and after 14 days at both protein and RNA levels. The three CpGs in the SRR2 region that were analysed in detail were found unmethylated at every time point tested during differentiation suggesting that no changes in methylation status are taking place at these sites. However, it cannot be excluded that other CpG sites in SRR1 and SRR2 not tested in this

study might have become methylated upon differentiation. Hence further studies extending the analysis of DNA methylation to all CpG sites within this region are required to determine whether DNA methylation within these regions plays any role in silencing of Sox2 under the differentiation conditions used.

MSCs are physiological precursors of bone cells and they were subjected to differentiate towards osteoblast lineage under similar conditions to that of ES cells with the objective to profile and then compare DNA methylation changes in cells derived from both adult and embryonic origin (chapter 5). Both SRR1 and SRR2 were found to be methylated at all time points in undifferentiated and differentiated MSCs when analysed by MS-PCR. Unfortunately it was not possible within the time frame of this study to analyse individual CpGs using bisulphite sequencing due to technical limitations (Chapter 5 section 5.3). As detailed earlier (chapter 3.4 and 4.4) that these regions have been studied and implicated in Sox2 expression in ESCs and neural stem cells so far, so could be possible that they are methylated in MSCs. Sox2 expression was detected only in undifferentiated MSCs and not in differentiated cells both at RNA and protein level so there is a possibility of DNA methylation leading to gene silencing. This seems quite interesting on one hand given that no data regarding DNA methylation is available about these regions in MSCs so far. But since this MSC cell line is immortalized hence, it cannot be excluded that methylation of these regions might be a consequence of cell line establishment and maintenance rather than actual physiological phenomena and hence need to be supplemented with data from primary cell types. Indeed some of the earlier studies have looked at and reported differences in gene expression patterns and DNA methylation in immortalized cell lines (Caliskan et al., 2011; Wild et al., 2010)

In summary, this is the first study looking at DNA methylation status of two regulatory regions of Sox2 i.e. SRR1 and SRR2 in undifferentiated mouse embryonic stem cells and upon their differentiation when grown in osteoblast and neural medium in a time course dependent manner. Similarly no study has so far described the DNA methylation profile of these regulatory regions of Sox2 in undifferentiated and differentiated mouse mesenchymal stem cells. Data obtained in this study hints towards the possible involvement of differential DNA methylation in regulating Sox2 expression in different cell types. There seems to be promising differences in methylation status of these two regulatory regions of Sox2 not only between cell types i.e. embryonic v/s adult but also lineages i.e. osteoblasts v/s neural though still preliminary and incomplete. Given the importance of epigenetic regulation in cell commitment and differentiation, analysis of these regulatory regions will provide a valuable insight into molecular mechanisms of gene regulation and how they contribute towards cell fate decisions and switch between different cell types. As these regions are highly conserved in mouse and humans, it could be proposed that regulatory mechanisms might also be shared hence making mouse embryonic stem cells a good model system to study regulation of Sox2 during in vitro differentiation.

7. Appendices

7.1.Chemicals, Medium and Reagents

Chemical Name	Catalogue No.	Supplier
Gelatin	G1393	Sigma
Bacterial petri dishes	633102	Greiner
Tri Reagent	T9424	Sigma
DNase	04536282001	Roche
RNasin	Promega N2511	Promega
Reverse Transcriptase	11785826001	Roche
Thermopol Buffer	B9004S	BioLabs
dNTPs	U1240	Promega
Taq polymerase	M0267L	BioLabs (NEB)
Orange G	03756	Fischer Scientific
Glycerol	G8773	Sigma
Phase lock tubes	129025	Qiagen
MspI	R0106S	BioLabs
HpaII	R0171S	BioLabs
EZ DNA methylation kit	D5006	Zymo Research
DMEM + glutamax	61965-026	Invitrogen Gibco
Fetal Calf Serum	EU-000-FE	SLI
MEM Non essential amino acids	11140-035	Invitrogen Gibco
Sodium pyruvate	11360-039	Invitrogen Gibco

Penicillin/Streptomycin	15070-063	Invitrogen Gibco
2-Betamercaptoethanol	M6250	Sigma
LIF	ESG1107	Millipore Chemicon
DMSO	C6295	Sigma
DMEM	21885-025	Invitrogen Gibco
Fetal Bovine Serum	16170-07	Invitrogen Gibco
Beta glycerol phosphate	410990250	Acros Organics
Ascorbic Acid Phosphate	A8690-54	Sigma
Dexamethasone	D4902	Sigma
Retinoic Acid	R2625	Sigma
DMEM:F12	21331-020	Invitrogen Gibco
N2 Supplement	17502-048	Invitrogen Gibco
Glutamax	35050-038	Invitrogen Gibco
Neurobasal Medium	21103-049	Invitrogen Gibco
B27 Supplement	17504-044	Invitrogen Gibco
Alizarin Red S	A5533	Sigma
Phenol	P4557	Sigma
Chlorofrom	C2432	Sigma
Sodium Acetate	9740	Sigma
Proteinase K	P2308	Sigma
Sox2 antibody	ab97959	abcam
Sox9 antibody	AB5535	Millipore

Osterix antibody	ab22522	abcam
Osteocalcin antibody	sc-30045	Sant Cruz Biotechnology
FITC conjugated anti rabbit anitbody	DI-1488-1.5	Vector Laboratoreis
Vectashield	H-1200	Vector Laboratoreis

7.2. Medium and Buffer Compositions

Complete ES medium

DMEM + glutamax (4.5g/l D-glucose + GlutaMax)

18% Fetal Calf Serum (v/v) (ES Grade)

1X MEM Non essential amino acids (100X)

1X Sodium pyruvate (100X)

1X Penicillin/Streptomycin (100X)

0.1mM 2-Betamercaptoethanol (100mM liquid)

1000U/ml LIF (10000000 U/ml)

Freezing Medium

90% Fetal Calf Serum (v/v)

10% DMSO (v/v)

EB Medium

DMEM (1g/l D-glucose + Pyruvate + GlutaMax)

15% Serum (not ES Grade)

1X Non essential amino acids (100X)

1X Penicillin/Streptomycin (100X)

0.1mM 2-Betamercapoethanol (100mM liquid)

Osteogenic Differentiation Medium

DMEM + glutamax (1g/l D-glucose + GlutaMax)

15% Fetal Calf Serum (v/v) (not ES Grade)

10mM Beta glycerol phosphate (1M stock in DMEM, filter sterilise at 4^{o}C for 1 month)

50µM Ascorbic Acid Phosphate (50mM stock in DMEM, filter sterilise at 4^{o}C for 1 month)

10 uM Dexamethasone (1mM stock in EtOH, keep at -20^{o}C)

1X Penicillin/Streptomycin (100X)

Retinoic Acid

5mM stock in DMSO (light sensitive)

N2 Medium

DMEM:F12

1X N2 Supplement (100X)

0.5X Glutamax (100X)

1X Penicillin/Streptomycin (100X)

Neurobasal Medium

Neurobasal Medium

1X B27 Supplement (50X)

1X Penicillin/Streptomycin (100X)

Cell Lysis Buffer

[Note: All chemicals were of analytical grade from Sigma, unless specified separately]

10mM Tris-HCl pH 8.0

10mM NaCl

10mM EDTA pH 8.0

0.5% SDS (w/v)

50µg/ml Protienase K (10mg/ml)

2X DMSO Buffer

20% DMSO (v/v)

32mM Ammonium Sulphate

20mM Betamercaptoethanol

134mM Tris-HCl pH 8.8

7.3.Primers Sequences for RT-PCR and MS-PCR

Gene	Primer ID	Primer Sequence	Product (bps)	Annealing Temperature
mAprt	mAprt1 (RT)	GGTAGCTCACAAAGGTCACT		
	mAprt2 (F) mAprt3 (R)	GGAAATCCAGAAAGATGCCT TCTAGCCAGCTCCTCAGTCA	227	60˚C
Gapdh	mGapdh(F)	CCCACTAACATCAAATGGGG	275	60˚C (Ying et al., 2008)
	mGapdh(R)	CCTTCCACAATGCCAAAGTT		
Sox2 (cDNA)	J11Sx2 (cDF)	CTGCGGAAAAAAACCACCAATC	241	62˚C
	J12Sx2 (cDR)	TTTGCGAACTCCCTGCGAAG		
Sox9 (cDNA)	J13 Sx9cDF	GAAGTCGGTGAAGAACGGACAAG	364 (973 on genomic DNA)	62˚C
	J14 Sx9cDR	GGGTGGCAAGTATTGGTCAAACTC		
Igf2 – DMR2 (MS-PCR)	M115 (F)	CGTGGCACTTTTGAGTTCATCTCTC	489	65˚C
	M114 (R)	ATTCTGGTTGTGCCGAGTTGCGAG		
mAprt (MS-PCR)	M116 (F)	TCTTCCCCGACTTCCCAATC	164	60˚C
	M117 (R)	GAACAAGGACAGGAGAGTGACCC		
CSa (MS-PCR)	CSa_s (F)	TGGTTGGCATTTTATCCCTAGAAC	133	59˚C (Weber et al., 2007)
	CSa_as (R)	GCAACATGGCAACTGGAAACA		
Runx2 (cDNA)	mRunx2_F	CCTGAACTCTGCACCAAGTC	234	64˚C (Kawaguchi et al., 2005)
	mRunx2_R	GAGGTGGCAGTGTCATCATC		
Osteopontin (cDNA)	mOsteopontin_F	TGCACCCAGATCCTATAGCC	186	64˚C
	mOsteopontin_R	CTCCATCGTCATCATCATCG		
Osteocalcin (cDNA)	mOsteocalcin_F	CCCTGCTTGTGACGAGCTAT	170	52˚C

	mOsteocalcin_R	ACTTGCAGGGCAGAGAGAGA		
Alk-P (cDNA)	mAlp_F	GAAGACGTGGCGGTCTTTGC	457	64˚C (Chen et al., 2006)
	mAlp_R	GGGAATCTGTGCAGTCTGTG		
Osterix (cDNA)	mOsterx_F	CCTCTGCGGGACTCAACAAC	355	64˚C (Kawaguchi et al., 2005)
	mOsterix_R	TGCCTGGACCTGGTGAGATG		
Nestin (cDNA)	mNestin_F	TCCCTTTCTCCCCCTTAAAA	198	52˚C
	mNestin_R	ACTGAGCAGCTGGTTCTGC		
Pax6 (cDNA)	mPax6_F	GGCTTTGTGCAAGATTCTCC	170	60˚C
	mPax6_R	CTCGGCCTAGTGGCTTCTTT		
Beta III tubulin	mβIIItubulin_F	AAGGTAGCCGTGTGTGACATC	209	60˚C (Ahn et al., 2004)
	mβIIItubulin_R	ACCAGGTCATTCATGTTGCTC		
Sox2 – SRR1	Sox2 – SRR1 – F	AAGTAACCCTGATGGTTTAAGCTC	536	62˚C
	Sox2 – SRR1 – R	ACGCAGCGTTCTCAGGAT		
Sox2 – SRR2	Sox2 – SRR2 – F	AAGGCACCAAGAACCAGAAA	419	62˚C
	Sox2 – SRR2 – R	GCCCGGGAAATTCTTTTAGA		

7.4.Primer Optimizations

All primer pairs were first optimized using temperature gradient, varied PCR buffers and Mg^{2+} concentrations to find optimal annealing and amplification parameters for PCRs. Below are some gel pictures showing various optimization experiments results.

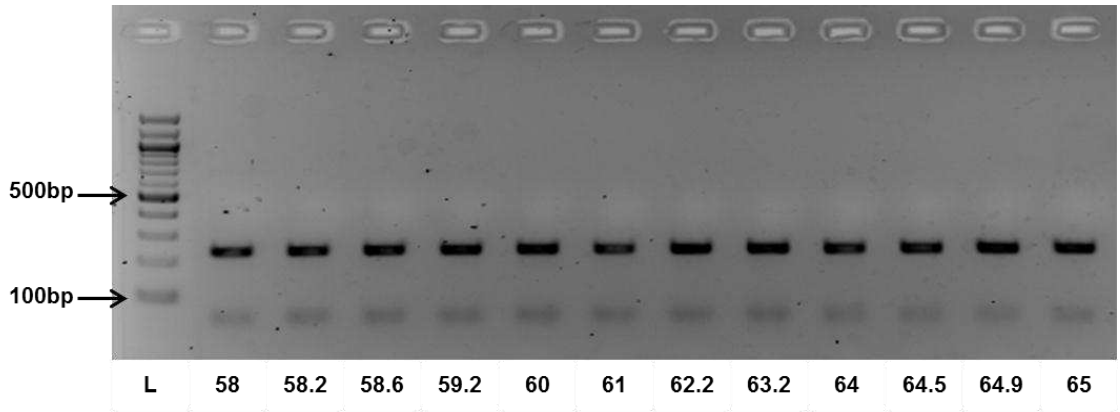


Figure 7.1: Temperature gradient (58-65˚C) for Sox2 (241bps) primer pairs

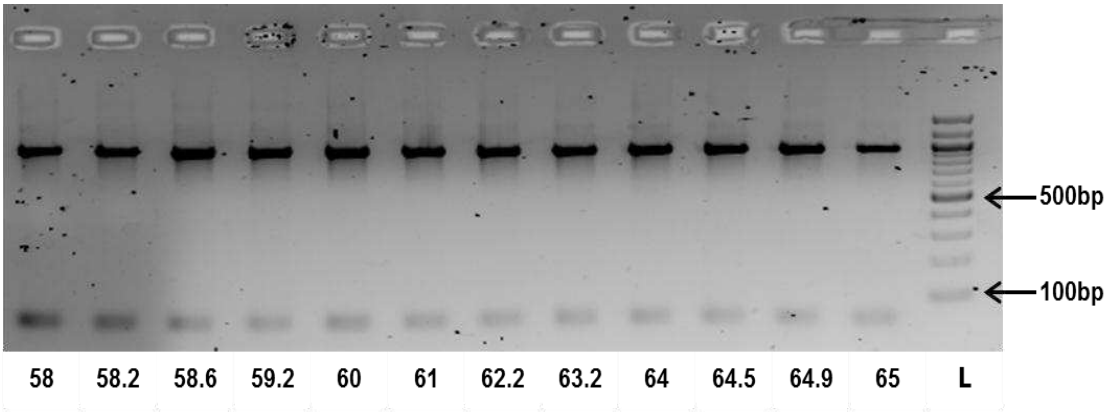


Figure 7.2: Temperature gradient (58-65˚C) for Sox9 (963bps) primer pairs

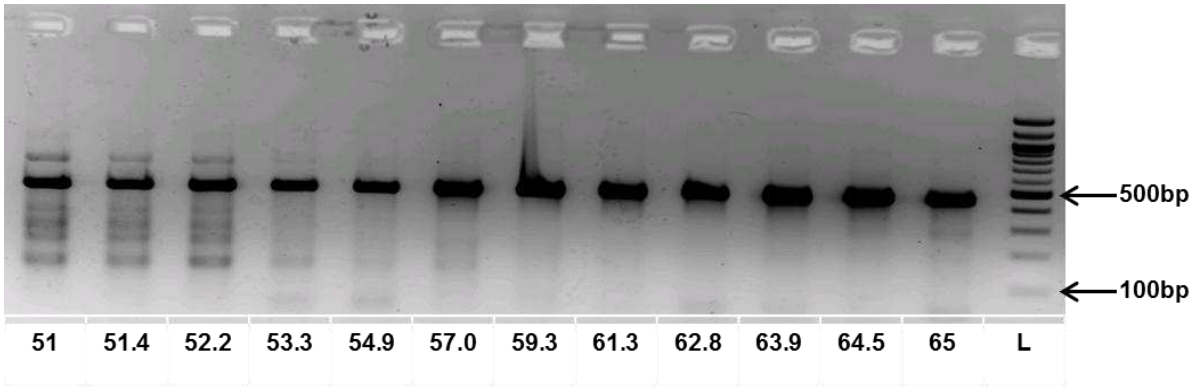


Figure 7.3: Temperature gradient (58-65˚C) for Alkaline phosphatase (457bps) primer pairs

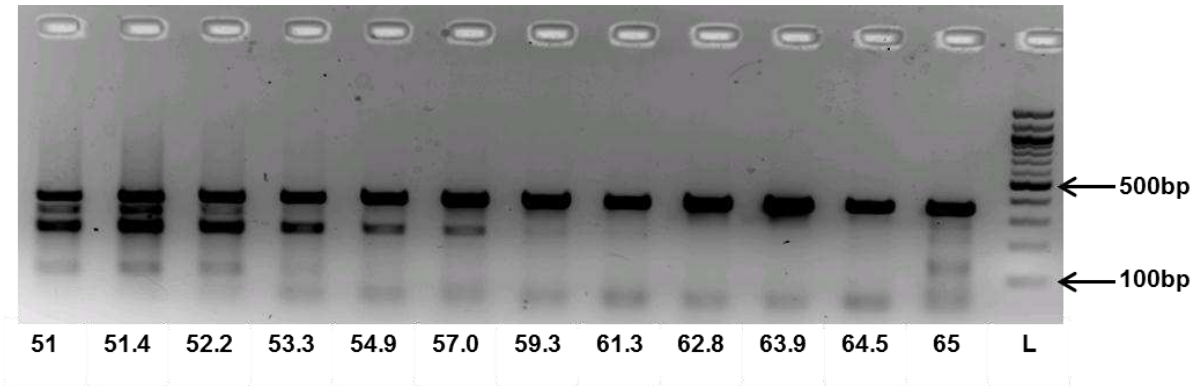


Figure 7.4: Temperature gradient (58-65˚C) for Ostetix (355bps) primer pairs

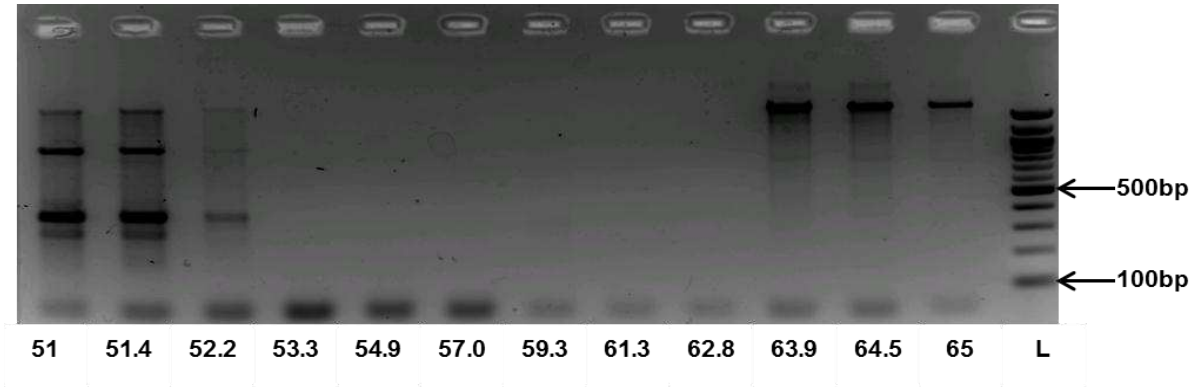


Figure 7.5: Temperature gradient (58-65˚C) for Osteopontin (1.4Kb) primer pairs

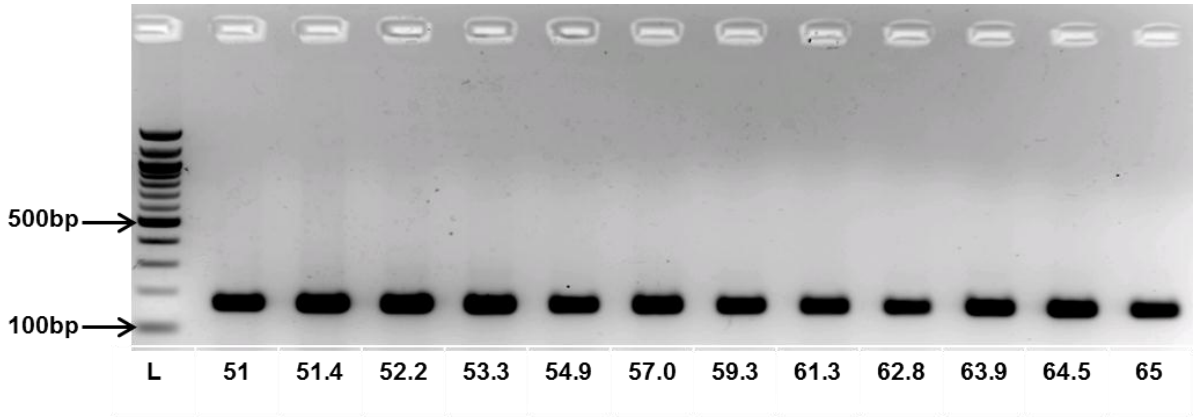


Figure 7.6: Temperature gradient (58-65˚C) for Osteocalcin (170bps) primer pairs

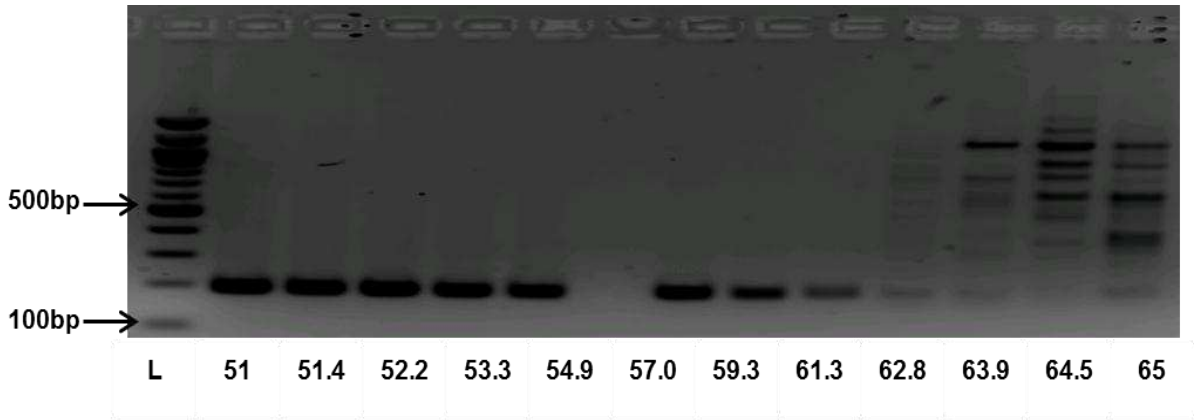


Figure 7.7: Temperature gradient (58-65˚C) for Nestin (198bps) primer pairs

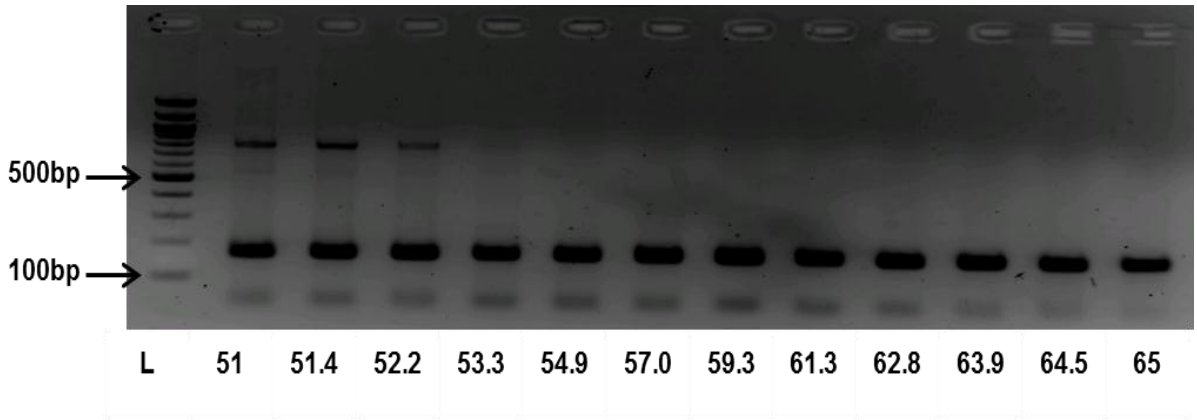


Figure 7.8: Temperature gradient (58-65˚C) for Pax6 (170bps) primer pairs

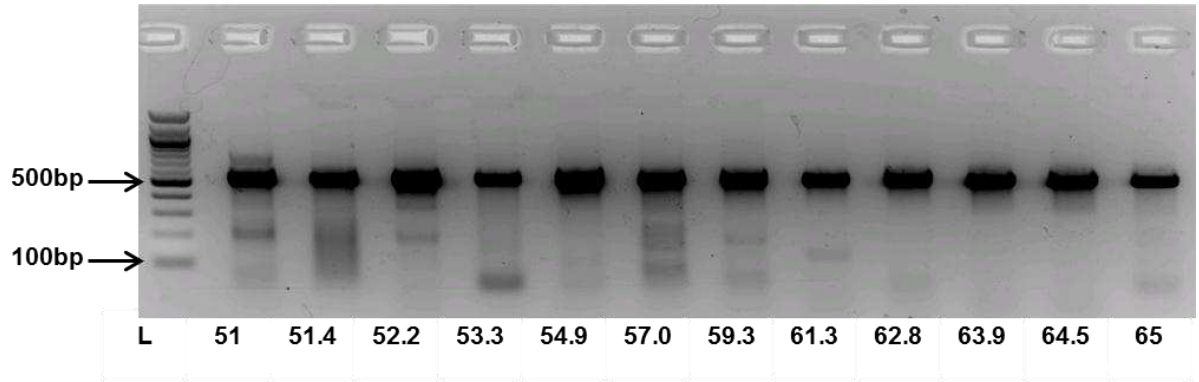


Figure 7.9: Temperature gradient (58-65˚C) for SRR1 (536bps) primer pairs

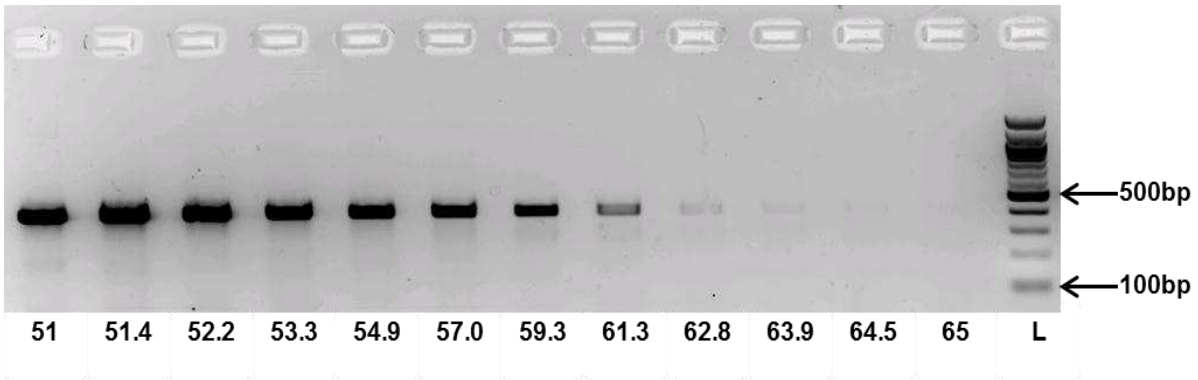


Figure 7.10: Temperature gradient (58-65˚C) for SRR2 (419bps) primer pairs

7.5.Sequencing Chromatograms

8. Bibliography

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