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THE AMERICAN UNIVERSITY IN CAIRO

الجامعة الأمريكية بالقاهرة

School of Science and Engineering

**Direct Conversion of Mouse Fibroblasts into
Osteoblasts and Investigating COBRA1
Expression Response**

A Thesis Submitted to

The Biotechnology Master Program

In partial fulfillment of the requirements for the degree of Master of
Science

By:

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Abstract

Fibroblasts are the most common connective tissue cells and are responsible for the synthesis of extracellular matrix (ECM) and collagen. They are mainly of mesenchymal stem cells (MSCs) origin. Fibroblasts are fully differentiated cells although some recent findings reported that fibroblasts possess some potency that allows them to differentiate into other cells types. They are said to undergo adipogenic, chondrogenic, hepatogenic, neurogenic and osteogenic differentiation if grown in the suitable conditions. In the normal physiologic environment, fibroblasts can also differentiate to epithelial cells, a phenomenon known as the mesenchymal epithelial transition (*MET*).

In the current research, we induced L929 mouse fibroblasts to differentiate into osteoblasts cells using the growth media containing Dexamethazone, Ascorbic acid and Beta-Glycerol phosphate which are the substances known to promote osteogenic differentiation. We have increased the concentration of dexamethazone than that used previously, and investigated the effect of this increase on the time of induction. We also investigated the levels of *Cobra1* (which is an integral member of the negative elongation (NELF) complex) mRNA in L929 that underwent osteogenic induction compared to the non-induced ones. We show a successful induction the onset of which is earlier than previously reported in the literature. Furthermore, to the best our knowledge our current data, for the first time, suggest a potential role of *Cobra1* in maintaining the plasticity of L929.

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List of Abbreviations

ATF4: Activating Transcription Factor 4

AP-1: Activator Protein 1

ASC: Ascorbic acid

ACTH: Adrenocortico Tropic Hormone

B-GLY: Beta-Glycerol Phosphate

BSP: Bone Sialo Protein

BMP: Bone Morphogenetic Protein

Col1: Collagen type 1

Cobra1: Cofactor of BRCA1

DEPC: Diethylpyrocarbonate

DEX: De xamethazone

DMEM: Dullbecco's Modified Eagle Medium

ERK: Extra cellular Related Kinase

ECM: Extra cellular matrix

EMT: Epithelial Mesenchymal Transition

HDACs: Histone Deacetaylases

KLF: Krupple-Like Factor

Lef : Lymphoid enhancer factor

miRs: Micro RNAs

MKP1: Mitogen-activated Protein kinase dephosphatase

MEF: Mouse Embryonic Fibroblasts

MSCs: Mesenchymal StemCells

MET: Mesencymal Epithelial Transition

MMP 13: Matrix Metaloprotein 13

NELF: Negative Elongation Factor

OPG: Osteoprotegerin

OSX, SP7: Ostrix

PTH: Para Thyroid Hormone

PK D1: Protein Kinase D1

RT: Reverse Transcriptase

SP: Specificity Protein

TAF: Tumor Associated Host Fibroblasts

TGF B: Transforming Growth Factor Beta

Tcf : T-cell factor

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Introduction

Fibroblasts are the most common connective tissue cells and are responsible for the synthesis of extracellular matrix (ECM) macromolecular structure that provides physiological support to tissues and organs allowing them to perform their functions. They are derived from mesenchymal stem cells (MSCs). And Like MSCs, fibroblast cells appear to possess multi-lineage differentiation capacity, albeit to a lesser extent (**Kasem, Tanom, 2011**). . In the presence of inducers of differentiation fibroblast cells were found to produce adipocytes, chondrocytes, and osteoblasts (**Kasem, Tanom, 2011**). By adding osteogenic molecules (Dexamethazone, Ascorbic acid and B-Glycerol phosphate) in the culture media fibroblast will differentiate into osteoblasts (**Jaroslawn et al., 2016**). Moreover, fibroblast cells are known to differentiate into epithelial cells; a phenomenon referred to as the mesenchymal epithelial transition (*MET*) which is essential for organ architecture during fetal organogenesis (**Dianbo et al., 2011**).

The source of different somatic cells for laboratory or clinical uses has been the pluripotent stem cells either embryonic (with all the concerned ethical debate) or induced in the laboratory (with all the relevant difficulties, high cost, and low yield). So using the capacity obtained by certain somatic cells to differentiate into other cell types may be beneficial.

Valuable information is present regarding how the fibroblasts are induced to differentiate into osteoblasts (**Kasem, Tanom, 2011**). This induction was done by growing fibroblasts in osteogenic media with a certain concentration that resulted in an extended period of time consumed by the fibroblasts to differentiate into fully mature and active osteoblasts that can mineralize the matrix (about two weeks). Changing the concentration of the osteogenic media used may result in earlier differentiation of the fibroblasts.

It is also unclear whether fibroblasts will attain any degree of potency before performing such differentiation. Here some questions arise and need to be answered;

- Are fibroblasts passing through a period of time in which the differentiation genes to any lineage are repressed
- Did the fibroblasts express any marker which its expression suppresses differentiation genes.

Or what is happening is direct change to other cell type by direct switching on and switching off certain differentiation genes.

Chapter (1): Literature review

1.1 Fibroblasts

Fibroblasts are connective tissue cells; they are responsible for synthesis of extracellular matrix and collagen. Fibroblasts are the principal active cells of connective tissue. They are large, flat, elongated (spindle-shaped) cells possessing processes extending out from the ends of the cell body. Fibroblasts produce tropocollagen, which is the forerunner of collagen, and ground substance.

The extracellular matrix (ECM) is a collection of molecules found between cells and secreted by them. It differs according to the type of connective tissues, in bone the extracellular matrix is composed of collagen fibers and mineralized bone tissue, in loose connective tissue it is composed of reticular fibers and ground substance while it constitutes the blood plasma. The structure of the extracellular matrix determines the physical properties of the connective tissues, and acts as a compression buffer against forces applied in the connective tissues (**Thomas and Janine, 2010**).

The extracellular matrix provides the structural and biochemical support to the surrounding cells; it plays an important role in cell division, cell adhesion, cell-to-cell communications and plays a critical role in wound healing (**Lauren et al., 2016**).

Fibroblasts are the most common cells in the connective tissue. Fibroblasts and fibrocytes are two different states of the same cell, the fibroblast is the activated state and they have different appearance according to state of activation and location. By secreting extracellular matrix which is composed of ground substance and fibers, fibroblasts maintain the structural integrity of the connective tissue.

The origin of fibroblasts like other connective tissue cells is the mesenchymal stem cell (MSC) which is an adult stem cell that can give rise to distinct group of cells (**César et al., 2011**). Fibroblasts can arise also from epithelium cells in certain cases in a phenomenon known as epithelial mesenchymal transition (EMT) (**Raghu and Robert, 2009**). In other situation the opposite can occur where fibroblasts may give rise to epithelial cells, which is known as mesenchymal epithelial transition (MET) (**Dianbo et al., 2011**) in certain circumstances as wound healing and tumor genesis.

Fibroblasts have branched cytoplasm, the nucleus has two or more nuclei, and abundant endoplasmic reticulum in active fibroblasts. Fibroblasts can migrate slowly as individual cell, and when cover large space, they are disjointed and scattered, but when crowded they are locally arranged in parallel clusters. In its active state as in growing individual and in wound healing the fibroblasts are dividing and secreting extra cellular matrix composed of collagen, glycosamino glycans, reticular and elastic fibers, glycoprotein found in the extracellular matrix and cytokines **(Lauren et al., 2015)**.

Fibroblasts play important role in the initiation of inflammatory process against an invading microorganism **(TA Wynn, 2008)**. Certain receptors in fibroblast surface induce chemokines synthesis, the immune cells will respond to chemokines to clear the invasive microorganisms. The fibroblast receptors also regulate blood formation and give rise for the immune cells to regulate fibroblasts.

Certain fibroblasts as the tumor associated host fibroblasts (TAF) play a critical role in immune regulation of tumors through the extracellular matrix components and modulators **(Michelle et al., 2017)**. The mouse embryonic fibroblasts (MEF) are used as feeder cells in stem cells researches.

1.2 Osteoblasts

Osteoblasts is another type of connective tissue cells and as fibroblasts they arise from mesenchymal stem cells **(Pittenger et al., 1999)**. They are responsible for bone formation. Osteoblasts are cells with single nuclei and function as a group of connected cells. The unit of bone is called the osteon and consists of a group of osteoblasts and the bone formed by them.

Osteoblasts synthesize mainly dense collagen type1 which constitutes about 90% of the bone organic matrix, it also secret specialized proteins such as Osteocalcin and Osteopontin in a much less quantity **(Bart, 2008)**. Osteoblasts then mineralize the organic matrix by depositing calcium and phosphorous in forms of hydroxyapatite, as the main component, as well as tricalcium phosphate and traces of many other salts. This mineralized matrix (bone) gives support to the body structure and acts as a mineral reservoir

Bone tissue is in constant state of flux, that means that it is a dynamic tissue that changes in shape and density in processes called modeling and remodeling (**Seeman, 2009**) in which constant alternative bone formation and bone resorption occur. The older bone, of high density and heavy weight relatively, which becomes brittle will be resorbed to give rise to deposition of newly formed bone with accepted density and hardness. If the resorption and deposition occur in the same site this is called remodeling which occurs in jaws bone, ribs and flat bones of the skull, on the other hand, modeling occurs in long bone of the skeleton where the resorption occurs from inside the bone (marrow side) and the deposition of newly formed bone occurs laterally on the external surface of bone.

Two types of bone cells are responsible for modeling and remodeling of bone, which are osteoblasts responsible for bone formation and osteoclasts responsible for bone resorption. The imbalance between osteoblastic and osteoclastic activities result in certain diseases as osteoporosis where there is increased activities of osteoclasts.

The osteoblasts are found in the periosteum outside the bone surface, and in endosteum layer at the inner surface of cortical lamellar bone and as a single layer of osteoblasts lining the mineralized tissue in trabecular bone and facing the osteoid bone tissue, which is the organic non-mineralized substances.

The outer osteoblastic layer is a layer of inactive flattened osteoblastic cells,. Another form of inactive osteoblasts is found where the osteoblasts are buried into the bone matrix to become the osteocytes, which are vital but inactive and remain connected to each others and to other osteoblasts through cell processes.

The bone formation and maturation (the osteogenesis) is done by one of two different processes, the endochondral ossification and the intramembranous ossification (**E.J.Mackie et al., 2008**). In endochondral ossification, bone is formed from cartilage, where resorption of cartilage is followed by bone formation, this ossification pattern is unusual. Whereas in the intramembranous ossification, there is direct ossification of the mesenchyme.

During osteoblastic differentiation, the osteoprogenitor cells express the regulatory, transcription factor Cbfa1/Runx2 and Sp7 transcription factor (**Tong , and Eng,**

2017) . The osteochondro progenitor cells differentiate under influence of growth factors. (**Arkady et al., 2016**)

Certain signaling molecules regulate osteogenesis, BMP (bone morphogenetic protein) is a key growth factor in endochondral skeletal differentiation; also, it regulates early skeletal patterning (**Maureen et al., 2013**). Transforming growth factor beta (TGF b) is a part of super family of proteins, which includes the bone morphogenetic proteins that have common signaling elements. Transforming growth factor beta is important in cartilage differentiation, which proceed bone formation in endochondral ossification. Another family of transforming growth factors determines where skeletal element occurs in relation to skin.

Parathyroid hormone plays an important role in osteoblastic regulation. Parathyroid hormone (PTH) increases the osteoblastic activities, while in high concentration it leads to degradation of bone matrix (**Lombardi et al., 2011**).

Estrogen and Glucocorticoids are important for reproduction and energy metabolism regulation of bone cells, while the Adrenocorticotrophic hormone (ACTH), in low concentration increases the activities of osteoblasts, while its increase leads to bone resorption. Its lack leads to osteosclerotic bone.

Individual osteoblasts are connected to each others by two types of connection, tight junction and gap junction (**Bart., 2008**).

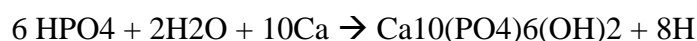
The bone organic matter is composed mainly of collagen type 1 protein, which is responsible for bone tensile strength. The osteoblasts secret collagen in two orientation and change the orientation of the collagen every few micrometers, osteoblasts deposit layer of collagen parallel to bone orientation alternative with layer perpendicular to bone orientation. The deficiency of collagen type 1 is inherited disorder of bone called osteogenesis imperfecta. Small amounts of Osteocalcin and Osteopontin bone proteins is deposited by osteoblasts to connect the organic and mineral contents of bone together. The Osteocalcin protein is not expressed except in bone tissue so it is a marker for bone matrix synthesis.

The bone mineralization occurs when calcium and phosphorous are deposited into the bone matrix (**Bart, 2008**), and due to the tight junction between the osteoblastic cells, the calcium and phosphorous cannot move in or out the bony compartment. The

calcium is transported across the osteoblasts by facilitated transport which means passive transport which does not pump calcium against gradient. The phosphorous in contrast is actively produced in form of phosphorous containing compounds like ATP. The osteoblasts secrete also phosphatases to cleave phosphorous to be free for mineralization formation.

The alkaline phosphatase protein is membrane anchored protein present at the surface of osteoblasts and expressed in large amount at the apical secretory face of active osteoblasts and considered as a characteristic marker for bone formation.

hydroxyapatite is formed of calcium, phosphate and water at slightly alkaline PH as following:



So, lowering the PH stops further precipitation. In case of cartilage, it presents no barrier for diffusion so the acid diffuse away, while in closed compartments the Na/H exchange removes the acid. In bone removal, reverse transport mechanism causes acid to be delivered to the mineralized matrix to drive hydroxyapatite into solution. The osteocytes have an important feedback mechanism as they secrete sclerostin protein when the osteon reaches a limiting size (**Mitchell et al., 2014**), this protein inhibits osteoblastic activity and inactivate bone synthesis. The osteoblasts have spherical and large nucleus with substantial presence of rough endoplasmic reticulum. In active osteoblasts, there is prominent Golgi apparatus, which appears as clear zone adjacent to nucleus.

1.2.1 Osteoblastic differentiation:

The bone marrow mesenchymal stem cells (MSCs) are multipotent stem cells and they are the origin of osteoblasts (**Jess et al., 2008**). They possess a highly proliferative capacity and can give rise to unipotent progenitor cells of many types as chondroprogenitor cells, osteoprogenitor cells and adipocyte progenitor cells. In other tissue types, the mesenchymal stem cells can give rise to other cell types such as myoblasts, hematocytes and neural tissues.

The osteoblasts differentiation passes through different stages (**Arkady et al., 2016**). The first is the inductive and proliferative stage in which the mesenchymal stem cells are committed to form osteoprogenitor cells that divide, migrate and end by the

formation of primitive osteoblasts. The second stage is the differentiation of osteoblasts and synthesis of extracellular organic matrix and full maturation of osteoblasts. The third stage is the mineralization of the organic matrix by the fully mature osteoblasts.

During these stages of differentiation from the mesenchymal stem cells to mature osteoblasts the cells are influenced by different factors, and each stage of differentiation is characterized by the expression of definite osteoblastic markers. The factors that influence the osteoblastic differentiation include: signaling protein and molecules, transcription regulation factors, growth factors and hormones. The following is a detailed description of these factors and their roles in osteogenesis:

1.2.2 Factors influencing osteogenesis

Runx2:

Runx2 is the master transcription factor that regulates osteogenesis. It acts through the induction, differentiation and maturation of osteoblasts (**Jess and Paul, 2008**). Many transcription factors and signaling pathways that influence osteogenesis do so by affecting *Runx2* expression and activities (**Banerjee et al., 1997**). The expression of *Runx2* is poorly correlated to the expression of its target gene as the *Runx2* protein passes through post translation modifications like phosphorylation, acetylation and ubiquitination which facilitate degradation of *Runx2* by proteasomes, (**Bellido et al., 2003**) alter *Runx2* activities or its cellular location.

Runx2 regulates the expression of many osteoblast genes (**Komori et al., 1997**), in addition to its DNA binding motifs, *Runx2* contains multiple domains for transcription co-activators or co-repressors (**Jess and Paul, 2008**).

Histone acetyl transferases such as P300, CBP, PCAF, MOZ and MORF are co-activator for *Runx2* (**Hecht et al., 2000**). They add acetyl group to lysine residues of histone and non-histone target proteins, and they alter protein-protein interaction and protein stability (**Pelletier et al., 2002**). In case of nucleosomal histone acetylation, this refers to opened chromatin structure which leads to more transcription activity of the target gene (**Sierra et al., 2003**). Opposite action is done by histone deacetylases (HDACs), (**Cho et al., 2005**) which leads to deacetylation of histone that results in more condensed chromatin structure and inhibits transcription of the target gene

(Geneviève et al., 2012). Inhibition of deacetylation at *Runx2* repression domain increases *Runx2* activities, this inhibition of deacetylation could be done by small molecule inhibitors or RNAi suppression thus enhances osteogenesis. On the other hand HDACs are co-repressors of *Runx2* as they bind *Runx2* and inhibit its activity (Jensen et al., 2007).

Multiple HDACs are co-repressors of *Runx2* as HDAC3,4,5,6 and 7, HDAC6 represses *Runx2* through its enzymatic activity, so inhibition of HDAC6 enzymatic activity can save *Runx2* from suppression effect of HDAC6 (Westendorf et al., 2002) while for HDAC7 (Jensen et al., 2007) this is not the case as it does not employ its enzymatic activity to repress *Runx2*, the mechanism of HDAC7 repression of *Runx2* is not clearly understood, however HDAC7 repression effect could be inhibited by BMP2 (bone morphogenetic protein 2) (Jeon et al., 2006) which activate protein kinase D1 (PK D1) that phosphorylate HDAC7 and leads to transient export of HDAC7 from nucleus so freeing *Runx2* from repression by HDAC7. Protein kinases can induce HDAC4, HDAC5 and HDAC7 to be exported from the nucleus even they exhibit different response to BMP2 stimulation (Vega et al., 2004).

HDACs proteins usually form large multi component repressive complex containing in addition to HDACs cofactors of HDACs such as NCoR, SMRT and Sin3a (Geneviève et al., 2012). The *Runx2* target genes expression is repressed by HDACs through different mechanisms and in response to various osteogenic signals including BMP2 and PTH (parathyroid hormone). At BSP (bone sialo protein) promoter, deacetylation of histone and subsequent repression of BSP transcription occurs as a result of recruitment of HDAC3 by *Runx2* at promoter site (Lamour et al., 2007).

BMP2 stimulates *Runx2* acetylation through SMAD dependent mechanism, SMADs are transcription factors that transduce extracellular TGF beta super family ligand signaling from cell membrane. *Runx2* acetylation protect *Runx2* from Smurf1 catalyzed proteolysis (Zhao et al., 2003). Smurf1 and Schnurri-WWP1 are ubiquitin ligases that leads to proteolysis of *Runx2*, *Runx2* acetylation by P300 is counter acted by HDAC4 and HDAC5 which remove the acetyl group from *Runx2* and thus brings *Runx2* to ubiquitin mediated proteolysis. BMP2 also stimulates the expression of estrogen related receptor which competes with P300 for *Runx2* thus preventing acetylation of *Runx2* and inhibiting BMP2 induced osteogenesis. (Abe et al., 2000).

Parathyroid hormone (PTH):

Parathyroid hormone (PTH) is another regulator of osteogenesis (**Lombardi et al., 2011**) which stimulates Runx2 interaction with acetyl transferases and induces Runx2 activity. Stimulation of osteoblasts by PTH leads to protein kinase A (PK A) dependent binding of P300 with Runx2 on the MMP13 (matrix metalloprotein 13) promoter resulting in histone acetylation and increased MMP13 gene expression. (**Bomah et al., 2009**). PTH also regulates Runx2 activity via different mechanisms such as phosphorylation and enhancing interaction with AP 1 transcription factor (**Ahlström and Lamberg., 1997**). On the other hand, PTH decreases Runx2 stability by stimulating ubiquitin mediated proteolysis limiting PTH stimulation of osteoblasts genes.

Osterix:

Osterix (Osx or SP7) is a zinc finger transcription factor expressed in osteoblast and as Runx2 is required for bone formation (**Jess and Paul, 2008**). It acts through the mineralization of bone, and it acts mainly in osteoblasts so the *Osx*-null mice lack intra membranous bone mineralization. *Osx* is down regulated by Runx2 which have a definite binding element on *Osx* gene promoter. *Osterix* activate collagen 1A1 promoter and this activation is enhanced by binding NFATc1 to *Osx*, the binding which is disrupted by *calcineurin*. *Osterix* also inhibit canonical Wnt signaling by inhibiting DNA binding to TCFs transcription factors.

ATF4 (activating transcription factor 4):

ATF4 has an important role in osteoblast formation. It is considered as a substrate for RSK2 which is serine/therotonin kinase essential for osteoblastogenesis, RSK2 mutation result in various skeletal abnormalities. *ATF4* combined with *Runx2* increases the transcription of *osteocalcin* and this is found to be activated by PTH signaling. Deficiency in *ATF4* results in decreased bone formation. *ATF4* also regulates energy metabolism in osteoblasts through decreased insulin production and responsiveness via altered *osteocalcin* and *leptin* endocrine signaling pathways (**Lee et al., 2007**).

SMADs:

SMADs are transcription factors interact directly with DNA and with other transcription factors as *Runx2* to induce bone formation (**Eric et al., 2010**). The *Runx2* domain for *SMAD* interaction has been identified and has been found to be continuous with the nuclear matrix target sequence. By *SMAD* activation of *Runx2*, the MSCs are induced to commit to the osteoblastic lineage through the induction of *Runx2* transcription. *SMADs* are activated through signaling by BMP and TGF beta family (transforming growth factors beta), they cause phosphorylation and nuclear translocation of receptor activated *SMADs* (*rSMADs*)

SMADs are inactivated by Smurf-directed ubiquitination which leads to proteolysis of *SMADs*. An interesting feedback loop is present among *Runx2*, *SMADs* and BMP that is BMP acts on *Runx2* to induce formation of *SMAD6* which is inhibitory for BMP activity and also leads to Smurf-directed ubiquitination of *Runx2* which leads to *Runx2* proteolysis, so via the two mechanisms *SMAD6* leads to decreased bone formation to prevent excess osteogenesis.

NFATc1/Calcineurin:

NFATc1 is a transcription factor which is highly phosphorylated and located in cytoplasm, it is dephosphorylated and imported to the nucleus by the effect of Calcineurin (**Hogan et al., 2003**) which is a phosphatase induced by intra cellular calcium signaling, import of *NFATc1* to the nucleus leads to transcription of its target genes (**Choo et al., 2009**). In osteoclasts, *NFATc1* increases the osteoclastic activities and bone resorption, while in osteoblasts it increases bone formation through activation of *Osterix* transcription. Over expression of *NFATc1* leads to decreased bone formation and maturation, it inhibits *TCF/LEF* transcription activity leading to reduced *osteocalcin* transcription via recruitment of HDAC3 to *osteocalcin* gene which ends in more condensed chromatin structure and decrease transcription.

Twist:

Twist is basic helix-loop-helix transcription factor which antagonize osteoblastogenesis, (**Bialek et al., 2004**). It binds to DNA at *Runx2* domain thus preventing *Runx2* from binding DNA, which results in inhibiting the transcription of

many osteogenesis genes. Twist also inhibits BMP/*SMAD* responsive transcription by forming a complex with *SMAD4* and HDAC1 (el Ghouzzi et al., 1997).

Activator protein (AP1):

AP-1 is a transcription factor composed of *Fos* related factors (*c-Fos*, *Fra1*, *Fra2* and *FosB*) and *Jun* protein (*c-Jun*, *Jun B* and *Jun D*). Multiple *Fos* and *Jun* proteins are highly expressed in proliferating osteoprogenitor cells (Grigoriadis et al., 1994) and decreases after that to the extent that only *Fra2* and *JunD* are the *AP-1* proteins found in mature osteoblasts (McCabe et al., 1996). At certain promoters *AP-1* interact with other transcription factors such as vitamin D receptor and *Runx2* to induce transcription of those genes (Grigoriadis et al., 1993), such genes like *osteocalcin*, collagenase-3, MMP 13, bone sialoprotein and alkaline phosphatase.

Tcf7/Lef1 transcription factors:

Tcf (T-cell factor) and *Lef* (lymphoid enhancer factor), these protein are nuclear effectors of canonical Wnt signaling (Ken and Marian, 2012). Activation of canonical Wnt signal transduction stabilize *beta-catenin* which translocates into nucleus (Birnbaumer., 1990) and associate with *Tcf7/Lef1* transcription factors, this association replaces HDACs and increases transcription of osteoblasts genes. Over expression of *Tcf7/Lef1* co-activators enhances expression of *osteoprotegerin* (OPG) which inhibits osteoclastogenesis and decreases bone resorption (Glass et al., 2005), while over expression of *Lef1* inhibits late state of osteoblastogenesis.

Zinc finger proteins:

Two main families of zinc finger transcription factors are present (Glimcher et al., 2007), the *Krupple-like factors* (*KLFs*), and specificity proteins (*SPs*). They interact with other transcription factors at promoters of target osteoblastic genes to increase transcription.

Micro RNAs (miRs):

These short non coding RNA ranging from 18-25 nucleotide binds to mRNA and leads to its degradation or prevents its translation, many of these miRs inhibits osteogenesis by repression of many osteoblast genes (Bantounas et al., 2004).

1.3 Osteogenic media:

The standard protocol for osteogenic differentiation of multipotent stem cells (bone marrow mesenchymal stem cells) is treatment of confluent mono layer of cells with a cocktail of Dexamethazone (Dex), Ascorbic acid (Asc) and Beta Glycerol phosphate (Beta Gly). These substances result in intracellular signaling cascades which lead to osteogenic differentiation (**Kasem and Tanom, 2011**).

Dexamethazone:

Dexamethazone induce *Runx2* expression by *FHL2/B-catenin* mediated transcription activation, *FHL2* protein activates *Wnt/B-catenin* signaling dependant *Runx2* expression. Dex binds to glucocorticoid response element on the *FHL2* promotor leading to increased *FHL2* expression. The *Wnt3a* (an activator of canonical *Wnt* signaling) leads to binding of *FHL2* to *Beta-catenin* which leads to transport of *Beta-catenin* into the nucleus where it bind to *TCF/LEF1* and leads to transcription of *Runx2*. In addition to *Runx2* expression, collagen type1 (*Col1*) is also up regulated (*Runx2* deficiency has no effect of *Col1* expression (**Hamidouche et al., 2008**)).

Dex signaling leads to transcription of *TAZ* which is a transcription activator that recruits the transcription machinery component for *Runx2* expression while in the same time *TAZ* represses transcription factors required for adipogenic differentiation. *TAZ* is crucial factor in osteogenic differentiation.

For *Runx2* activation and its binding to *TAZ*, it needs to be dephosphorylated. This dephosphorylation is done by the action of mitogen-activated protein kinase dephosphatase (MKP 1) whose expression is induced by Dex signaling. MKP1 dephosphorylate the serine resedue 125 of *Runx2*. After *Runx2* combination with *TAZ*, it needs to be phosphorylatd again on other serine resedues other than the serine125, this is done through cascade of events leading to *Runx2* phosphorylation without the need of Dex, however Dex increases the phosphorylation of *Runx2* through its up regulation of *Col1* whose secretion and the subsequent extracellular presence together with extracellular phosphorous can positively affect the signaling that leads to *Runx2* phosphorylation (**Hamidouche et al., 2008**)).

Ascorbic acid:

Ascorbic acid induces osteogenic differentiation by increasing *Col1* secretion (Langenbach, Handschel, 2013), Asc is considered as co-factor for enzyme that hydroxylate proline and lysine in pro-collagen, (Chatterjee., 1973) without this hydroxylation, the collagen chain cannot attain the helical structure required for collagen secretion. So the main function of Asc is to facilitate *collagen 1* secretion to the extra cellular matrix (Chojkier et al., 1989). For osteoblasts to differentiate it should be in contact with *collagen1* (Kuivaniemi et al., 1991) containing extra cellular matrix, osteoblasts communicate with the extracellular matrix through binding of *collagen1* to *alpha2 beta1 integrin*, this binding activates the MAPK signaling and related pathway which ends in phosphorylation of *Runx2* and expression of osteoblasts genes.

Beta Glycerol phosphate:

Beta Glycerol phosphate is considered as a source of phosphorous for bone minerals (Langenbach and Handschel., 2013) and also for phosphorylation of extracellular related kinase (ERK). The inorganic phosphates enter the cell and activate ERK signaling pathway, this activation is biphasic, first phosphorylation of ERK is followed after few hours by second phosphorylation. ERK is not active unless the second phosphorylation occurs. Activation of ERK pathway leads to phosphorylation of *Runx2* after its combination with *TAZ* and increase expression of osteoblastic genes, ERK pathway also induce the transcription of *Osteopontin* bone protein and the phosphate related expression of BMP2 which enhances osteogenesis. The inorganic phosphate regulate BMP2 expression via cyclic AMP/protein kinase A pathway, which is independent of ERK phosphorylation pathway (Langenbach, Handschel, 2013).

1.4 Fibroblastic differentiation into osteoblasts:

Using the differentiation media containing cocktail of osteogenic substances (Dexamethazone, Ascorbic acid and Beta Glycerol phosphate) not only the stem cells whatever their potency capacity are able to differentiate into fully mature and active osteoblasts (Jaroslaw et al., 2016), but also fully differentiated somatic fibroblasts are able to differentiate into osteoblasts (Kasem, Tanom, 2011).

Fibroblasts have the capacity to differentiate into different types of cells using the suitable differentiation media, this differentiation capacity differs according to the source of fibroblasts; the species which the cells were driven from and the tissues origin that cells have originated from. So beside fibroblastic differentiation into osteoblasts, fibroblasts are able to undergo adipogenic, chondrogenic, neurogenic and hepatogenic differentiation (**de Crombrughe et al., 2000**).

Valuable information are present regarding how the osteogenic substances can induce osteogenic differentiation to the fibroblasts (**Kasem, Tanom, 2011**), but this differentiation consumed a long time which is considered as a limitation of the induction mechanism. It is also unclear whether fibroblasts will attain any degree of potency before performing such differentiation, that means: are fibroblasts passing through a period of time in which the differentiation genes to any lineage are repressed and the fibroblasts possessed any potency characters which prevent differentiation or expressed any marker which its expression suppresses differentiation genes, or what is happening is direct change to other cell type by direct switching on and switching off certain differentiation genes without passing through any degree of potency. The potency of cells are achieved through two different mechanisms, first of them is the maintenance of potency characters and the second mechanism is differentiation prevention.

1.5 *Cobra1*:

Cobra1 is the B subunit of NELF (negative elongation factor) and is known as NELF-B. It is one of four subunits that constitute this protein, it was considered as co-factor of *BRCA1* but it is found to regulate genes which are regulated by *BRCA1* in absence of *BRCA1* (**Amleh et al., 2009**).

Cobra1 acts interdependently with NELF component to repress the transcription of genes responsible for cellular differentiation through pausing RNA polymerase and stopping the elongation of the transcript.

Cobra1 in pluripotent cells is maintaining pluripotency through prevention of differentiation (**Jaroslawn and Maciej, 2016**), it does not repress the transcription of master genes responsible for pluripotency such as *Oct4*, *Nanog* and *Sox2*, but mainly regulate differentiation genes although it represses *Tcf3* transcription factor that

occupies areas of promoters of the master pluripotency regulators, so plays a role in maintaining pluripotency, however the pluripotency master genes can keep pluripotency in absence of *Cobra1* which means that the main function of *Cobra1* is the prevention of differentiation rather than directly affecting the master regulators of pluripotency.

Cobra1 deficiency in multi potent stem cells, unipotent progenitor cells and somatic cells may result in tumor formation, and the cells that lack *Cobra1* exhibit high tendency of differentiation (**Amleh et al., 2009**).

Cobra1 null embryonic stem cells can not develop to an embryo and experience lethality although the presence of master pluripotency proteins, and exhibit premature differentiation.

Cobra1 acts mainly through pausing transcription of Lef1 so inhibits Lef1/Tcf-mediated Wnt/Beta-catenin signaling pathway that is important pathway in transcription of many differentiation genes (**Ken and Marian, 2012**).

So, *Cobra1* prevents differentiation of ESCs and inhibits further differentiation machinery in somatic cells, and down regulation of *Cobra1* in somatic cells may permit the differentiation machinery of the cells to resume according to the induction mechanism.

1.6 Collagen1

Collagen type I is the most abundant form of collagen found in bone. It is synthesized by osteoblasts during bone formation and considered as the major component of the bone organic matrix. It is considered as a marker of bone formation (**Arkady et al., 2016**).

1.7 Osteocalcin

Osteocalcin is a hydroxyapatite-binding protein exclusively synthesised by osteoblast, odontoblasts and hypertrophic chondrocytes. One of the major function of Osteocalcin is being responsible for the calcium binding. Osteocalcin can also interact with other proteins, including cell surface receptors. These functions predispose Osteocalcin as a molecule active in the organization of the extracellular matrix.

Study objectives

Regulation of certain genes transcription can induce a change in the cell type when these genes are responsible for differentiation into another fate of the cells. The somatic mouse fibroblasts L929 could be differentiated into mature mouse osteoblasts which possess all features of bone forming cells, including the production of osteogenic factors such as *Collagen1* and *Osteocalcin*, (**Delmas et al., 1986**) and bone minerals. These changes in the cells could be induced using osteogenic differentiation media containing Dexamethazone, Ascorbic acid and Beta Glycerol phosphate (**Kasem, Tanom, 2011**)., Before and after osteogenic induction, the cells could be examined for the levels of *Cobra1* expression to determine if it plays a role in osteoblastic differentiation, or whether the osteoblastic differentiation mechanism affects *Cobra1* regulation.

The aim of our study is a direct conversion of mouse fibroblasts into osteoblasts using osteogenic differentiation media, and further to analyze the levels of osteogenic differentiation factors (*Runx2*, *Collagen1*, and *Osteocalcin*) and a known pluripotent related gene (*Cobra1*).

Chapter (2): Materials and Methods

Experimental design

Mouse fibroblasts L929 were used for this analysis, study cells were incubated with osteogenic differentiation media containing Dexamethazone, Ascorbic acid and Beta Glycerol phosphate for 21 days during which the media were changed every 3 days, Alizarin red stain was applied to monitor the deposition of calcium in the induced and un-induced L929 cells after 2 and 3 weeks from the onset of induction. The same timetable has been followed by taking aliquots of control and study cells and processing them for RNA extraction followed by cDNA synthesis and PCR amplification using primers for specific osteoblasts genes (*Runx2*, *Osteocalcin* and *Collagen1*) (Ericet al., 2010), *Cobra1* gene primers and primers of *B-actin* as a control. The products of RT-PCR were run on a gel, and transcription analysis was done.

2-1 cell culture

Mouse fibroblasts L929 cell line was used in this study, the cells in passage 7 were saved frozen in liquid nitrogen at cell culture lab (Biology department, American university in Cairo).

Culture media (non osteogenic normal media)

The culture media was composed of; DMEM (Dulbecco's Modified Eagle Medium) (Lonsa, USA) supplemented with 10% FBS (Fetal bovine serum) (Lonsa, USA) and 5% Penicillin-streptomycin antibiotic (Lonsa, USA).

Differentiation media (osteogenic media)

It was composed of normal culture media supplemented with Dexamethazone 0.7 μ M (Sigma Aldrich) (in previous researches, the concentration used was 1 μ M), B-Glycerol phosphate 10 mM (Sigma Aldrich) and Ascorbic acid 0.2 mM (Sigma Aldrich). All the media were sterile filtered. Before the beginning of the experiments, the L929 fibroblasts were cultured in normal non osteogenic media, incubated at 37°C in humidified air mixed with 5% CO₂ and passed through passage 8 and passage 9.

At the beginning of the experiment, the cells were seeded in 6 well plates at density of 12500 cell per well (**Kasem, Tanom, 2011**), included in 3mL media in each well. The control cells were cultured in normal non osteogenic-media, while the rest of cells were cultured in differentiation osteogenic media, aliquot was taken from the fibroblasts in normal media at the beginning of the experiments to be used for RT-PCR, and photos were taken to the cells under inverted microscope. All the cells were incubated at 37°C in humidified air containing 5% Co₂.

The media were changed every three days and half, and photos for cells in different media were taken immediately before every media change. After two weeks and three weeks from the beginning of the induction (at day 14 and day 21), aliquots were taken from the control cells and the cells cultured with differentiation media for RT-PCR. The cells were stained using 2% Alizarin red S solution (Biostain ready reagents ltd., United Kingdom) (**Kasem, Tanom, 2011**), and photos were taken after staining using inverted microscope.

2-2 Alizarin red S staining

After 14 days and 21 days, the cells were fixed using 4% Paraformaldehyde (Minsing et al., 2011), washed and stained using Alizarin red S solution (Biostain ready reagent ltd., United Kingdom), later photos were taken using inverted microscope.

2-3 RNA extraction and quantification

RNA was extracted from cells using Trizol reagent (Invitrogen, USA) as recommended in the manufacturer protocol. The extracted RNA pellet was dissolved in 20µl DEPC (diethylpyrocarbonate) treated water. The resultant RNA solution was further diluted in DEPC in ratio 1:50 (2 µl of RNA solution and 198 µl DEPC) for quantification of RNA. The RNA samples were quantified by measuring the absorption at 260nm (A_{260nm}) using spectrophotometer (Shimadzu, Japan) and the concentration of each RNA sample was calculated. The purity of the RNA samples was detected by calculating the ratio of A_{260nm} and A_{280nm} and data were recorded.

2-4 cDNA synthesis using reverse transcriptase enzyme

Complementary DNA (cDNA) synthesis was done for each RNA sample using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) from 1 µg of the extracted RNA according to the manufacturer protocol. This was done using random primers, reverse transcriptase, dNTPs, RNase inhibitor, buffer and nuclease free water to the RNA samples. The cDNA synthesis was run using PCR machine according to manufacturer instructions.

2-5 Polymerase Chain Reaction (PCR)

RT-PCR (Reverse Transcription Polymerase Chain Reaction) was carried out for all the synthesized cDNA samples. Specific primers were used to test the presence of mRNA for the target genes. The primers used were: Runx2, Collagen1, Osteocalcin, Cobra1 and Brta-actin (House keeping gene) as an internal control. The sequences of the used primers and the amplicon sizes are mentioned in table (1), while the locations of the primers in respect to the exons are listed in table (2). The PCR reaction was carried out for each tested gene using a mixture consisting of 1 µl cDNA template, 5 µl 10X Dream Taq Green Buffer containing dNTP mix (Thermo Scientific), 0.25 µl Dream Taq DNA Polymerase (Thermo Scientific), 0.75 µl of each forward and reverse primers and 17.25 µl nuclease free water to reach final volume of 25 µl. The PCR conditions were optimized for each gene and mentioned in table (3).

The amplified PCR product was run on 2% agarose gel electrophoresis and visualized using Gel Doc EZ System (Bio-Rad, USA). The samples loaded onto gel were as following: 100 bp ladder, PCR product free of cDNA template but contained Reverse Transcriptase during cDNA synthesis, water free of any impurities, PCR products of the cDNA synthesized from RNA extracted from fibroblasts which were cultured without differentiation osteogenic media at day 1, day 14 and day 21, in addition to their control (samples containing no Reverse Transcriptase during cDNA synthesis), in addition to PCR products of the cDNA synthesized from RNA extracted from fibroblasts which underwent culturing in differentiation osteogenic media at day 14 and day 21, in addition to their control that had no Reverse Transcriptase during cDNA synthesis.

2-6 ImageJ software and Statistical analysis

The intensities of the bands in PCR products in the reactions concerning Runx2, Collagen1, Osteocalcin and COBRA1 were measured and normalized to the Beta-actin, which was used as an internal control. The presented data for Runx2, Collagen1 and Osteocalcin were averages \pm standard deviation (SD), the mean SD was used to produce the error bar for each gene result and the P-value for significance was calculated. A P-value less than 0.05 was considered significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Table 1: Sequences of primers used in the Reverse Transcription PCR and the genes amplicon sizes
(F: forward primer, R: reverse primer, bp: base pair)

| Gene | Sequences | Amplicon size |
|--------------------|--|---------------|
| <i>Runx2</i> | F: 5'-GATGGGACTGTGGTTACTGTCA-3' R: 5'-CTCAGATCGTTGAACCTTGC-3' | 110 bp |
| <i>Collagen1</i> | F: 5'-CAAGAACCCCAAGGACAAGAG-3' R: 5'-CTTGCAGTGGTACCTGATGTTC-3' | 169 bp |
| <i>Osteocalcin</i> | F: 5'-CTCACACTCCTCGCCCTATTG-3' R: 5'-GCCTGGGTCTCTTCACTACCT-3' | 142 bp |
| <i>Cobra1</i> | F: 5'-ACATCACCAAGCAGAGGAA-3' R: 5'-GATCCAGCTGTTCCAGCTTC-3' | 366 bp |
| <i>Beta-actin</i> | F: 5'-GCAAAGACCTGTACGCCAAC-3' R: 5'-GAGACCAAAAAGCCTTCATACATCTC-3' | 777 bp |

Table (2): Locations of the primers in respect to the exons

| Primers | Location in respect to exons |
|--------------------|--|
| <i>Runx2</i> | Primers are located on two different exons, exon 1 and exon 6. |
| <i>Collagen1</i> | Primers are located on two different exons, exon 1 and exon 51 |
| <i>Osteocalcin</i> | Primers are located on two different exons, exon 1 and exon 4 |
| <i>Cobra1</i> | Primers are located on two different exons, exon 1 and exon 13. |
| <i>Beta-actin</i> | Primers are located on one exon, as the gene contains one exon only. |

Table (3): PCR conditions for each gene

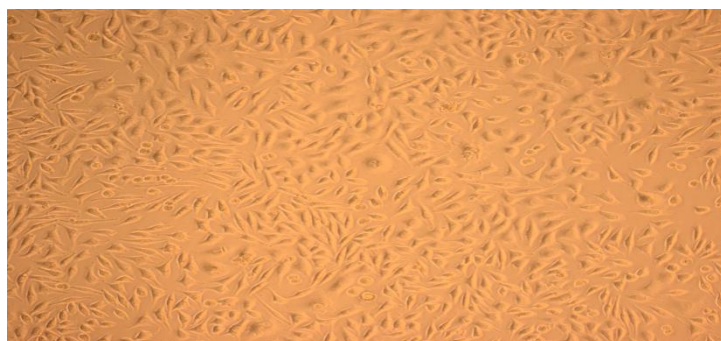
| Gene | Denaturation | Annealing | Number of cycles | Elongation |
|--------------------|--------------|--------------------------------------|------------------|------------|
| <i>Runx2</i> | 95°/10 min | 95°/10 sec, 60°/20 sec, 72°/20 sec | 40 | 72°/7 min |
| <i>Collagen1</i> | 95°/2 min | 95°/5 sec, 56°/20 sec, 72°/20 sec | 40 | 72°/7 min |
| <i>Osteocalcin</i> | 95°/2 min | 95°/30 sec, 60°/30 sec, 72°/30 sec | 40 | 72°/7 min |
| <i>Cobra1</i> | 95°/5 min | 95°/10 sec, 59.5°/20 sec, 72°/20 sec | 23 | 72°/7 min |
| <i>Beta-actin</i> | 95°/5 min | 95°/10 sec, 58°/20 sec, 72°/20 sec | 28 | 72°/7 min |

Chapter (3): Results

3-1 L929 cells grown in osteogenic differentiation media exhibit osteoblast-like phenotype within one week of culture:

The morphology of the studied cells has been examined microscopically at regular periods. As previously reported, the L929 cells that were cultured in growth media that did not contain osteogenic differentiation substances (control cells) exhibited heterogeneous morphology, including mainly spindle-like, some epithelial-like, and few round shaped cells during the whole period of the study (21 days). There was a noticed increase in the number of round shaped cells as the confluency of cells increased by time (Figure-1).

The cells that were grown in the osteogenic differentiation media developed morphological changes that became apparent after 84 hours from the beginning of culturing with the differentiation media. Cells assumed a triangular to cuboidal shape (Figure-2). By the end of the incubation period (21 days), the vast majority of cells exhibited the cuboidal shape.

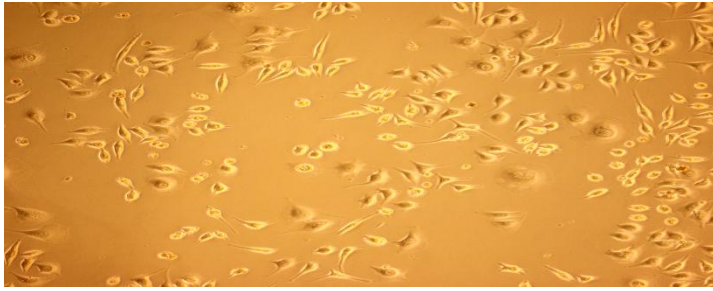


(A) 10x magnification

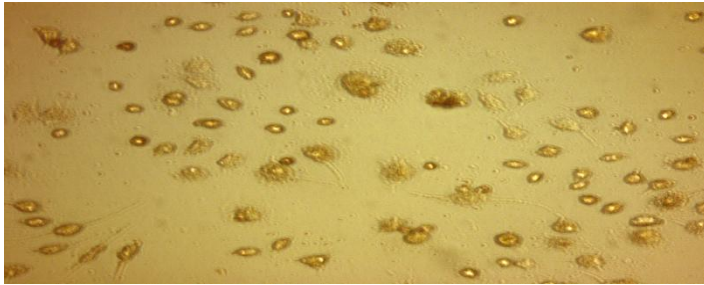


(B) 10x magnification

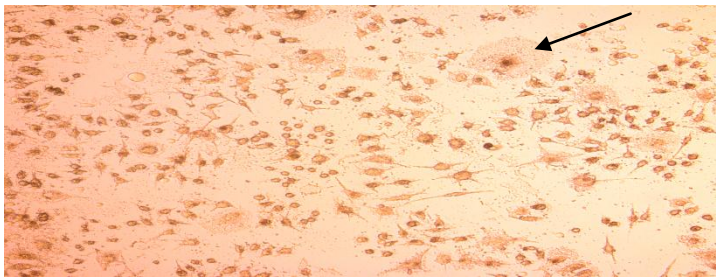
Figure-1: L929 fibroblasts in normal growth media (A) at day 1 of the experiment (B) at day 21 of the experiment. (10x magnification)



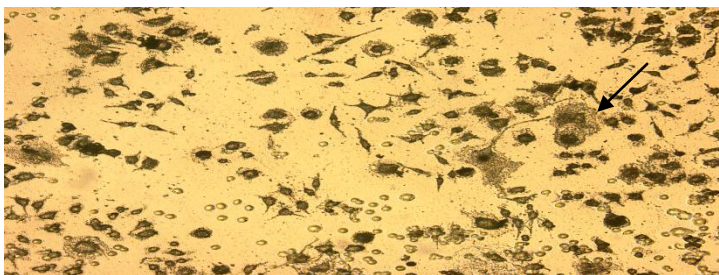
(A) 20x magnification



(B) 20x magnification



(C) 10x magnification

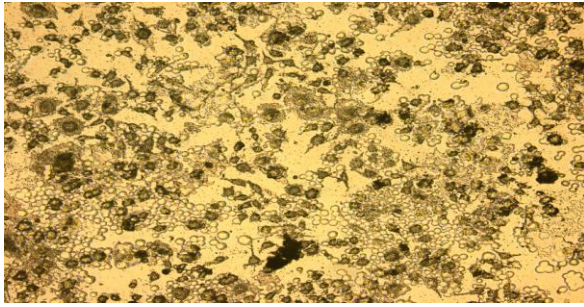


(D) 10x magnification

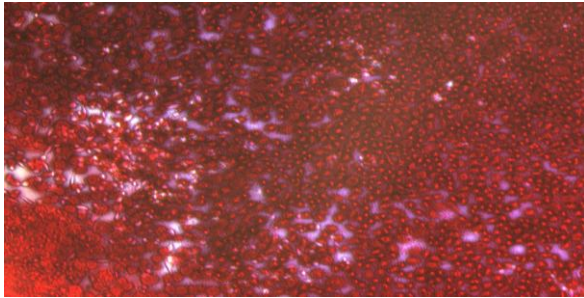
Figure-2: L929 fibroblasts grown under differentiation osteogenic media (A) after 84 hours, (B) after 1 week, (C) after 2 weeks and (D) after 3 weeks. (A and B: 20x magnification, C and D: 10x magnification). Arrows are pointing deposits around cells.

3-2 L929 cells cultured in osteogenic differentiation media produced calcium deposits as indicated by Alizarin red S staining:

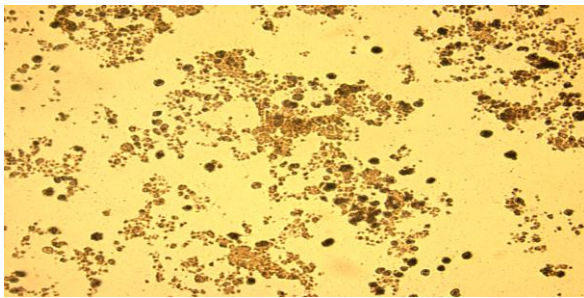
After two weeks from the start of the experiment, L929 fibroblasts were stained with Alizarin red S staining to monitor calcium deposition in osteoblasts. The control cells were slightly reddish, while cells that were cultured in differentiation osteogenic media were stained darkly with the bright red color indicating the presence of calcium deposits (Figure-3). After three weeks, almost no staining was observed in control cells, while cells in differentiation media were stained darkly with the bright red color (Figure-3).



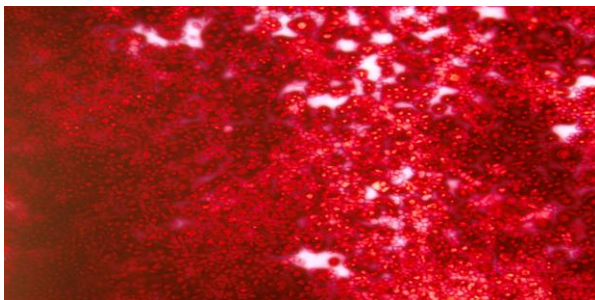
(A) 10x magnification



(B) 10x magnification



(C) 10x magnification



(D) 10x magnification

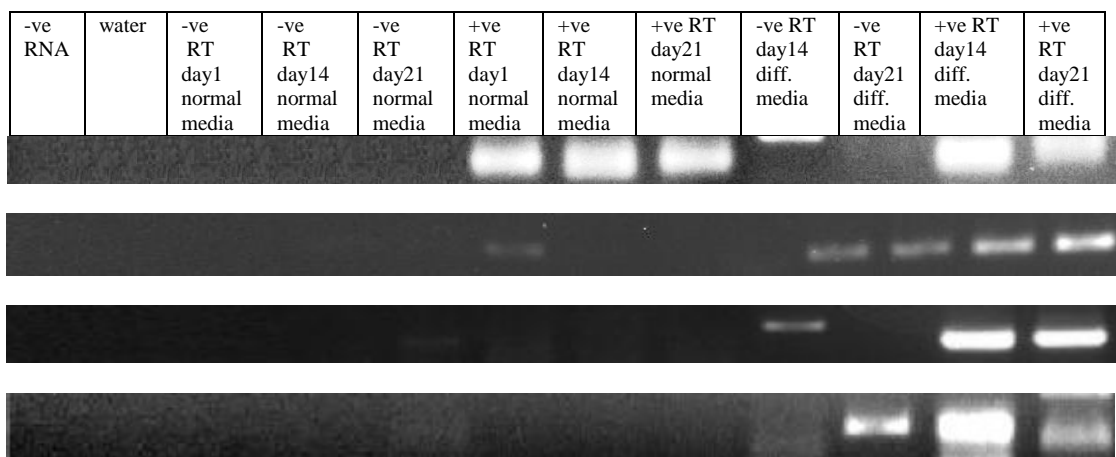
Figure-3: Alizarin red S staining after 2 weeks and from the beginning of the experiment: (A) fibroblasts L929 in normal growth media, (B) fibroblasts L929 grown in differentiation osteogenic media, and after 3 weeks: (C) fibroblasts L929 in normal growth media, (D) fibroblasts L929 grown in differentiation osteogenic media. (10x magnification)

3-3 L929 cells cultured in osteogenic differentiation media demonstrated osteoblast-like features as indicated by the expression of osteogenic factors (*Runx2*, *Collagen1*, and *Osteocalcin*) at the RNA level:

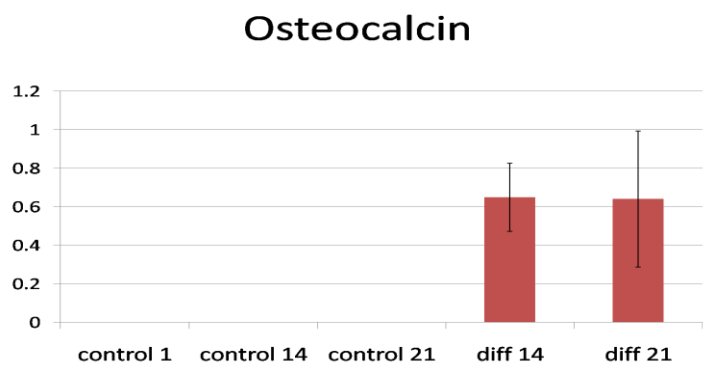
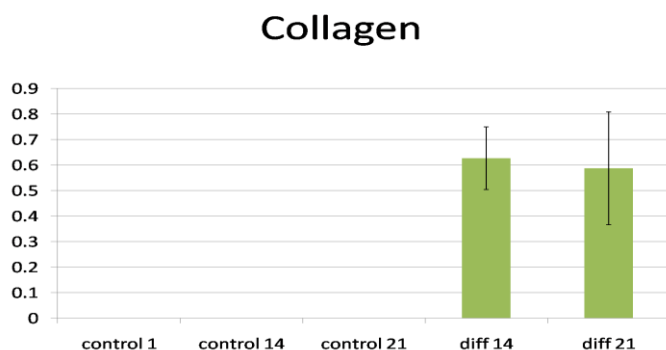
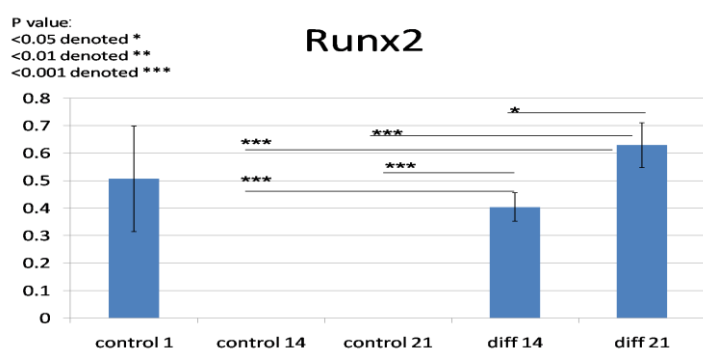
The mRNA level of the genes was examined using reverse transcription PCR for RNA extracted from the L929 cells that were cultured in regular media and those that were induced to differentiate into osteoblasts at different intervals (day1, after two weeks and after three weeks from the beginning of the experiment). Two negative control reactions, one containing no reverse transcriptase and the second containing no template were included in each reaction.

3-3-1 Levels of *Beta-actin* (house keeping gene) mRNA expression under different media at different periods of times: (to be used for examining PCR reaction validity).

Beta-actin gene expression was prominent in all the positive RT samples either for the cells which were grown in regular non-osteogenic media at all-time intervals (day1, day14, and day21) and for cells grown under differentiation osteogenic media at two distinct time intervals (day14 and day21). Figure-4 accounts for the PCR analysis for *Beta-actin* gene expression.



(A)



(B)

Figure-4: Osteogenic factors were detected in L929 induced cells. A) PCR analysis for transcription

of *Beta-actin*, *Runx2*, *Collagen* and *Osteocalcin* respectively from top to bottom.

***B-actin*:** The transcription was abundant in all lanes containing reverse transcriptase enzyme in cells cultured in regular or differentiation media at all-time intervals. The size of the bands is 777 bp, which is indicative for *Beta-actin* gene.

***Runx2*:** Thick bands in lanes of cells grown under differentiation osteogenic media, band after 21 days is denser than that of bands after 14 days. Faint band appears in lane representing cells in normal media at day 1. The size of the bands is 110 bp indicative for *Runx2* gene.

***Collagen1*:** Abundant bands representing *Collagen1* gene presence in lanes of cells grown under differentiation osteogenic media after 14 days and 21 days. The 14 days band is denser than that of 21 days. The size of bands is 169 bp that is indicative for *Collagen1*.

***Osteocalcin*:** Dense band for cells grown in differentiation media at day 14, and a faint band for the same cells under the same media at day 21. A band for *Osteocalcin* is present also in lane of negative RT samples at day 21, which may indicate DNA contamination of this sample. The size of the band is 142 bp, which is indicative for *Osteocalcin* gene

B) Graphical representation for the transcription levels of *Runx2*, *Collagen1* and *Osteocalcin* by ImageJ, normalized to *Beta-actin*. Error bars and P-value for significance are represented. The *Runx2* expression is significant in differentiated cells compared to the control cells after 14 and 21 days ($***P < 0.001$). The expression of differentiated cells after 21 days is significant compared to that after 14 days ($*P < 0.05$).

3-3-2 Levels of *Runx2* mRNA expression under different media at different periods:

The *Runx2* expression was abundant in the cells grown in differentiation osteogenic media. This is presented by dark bands in their lanes; however, the bands of the cells after 21 days are thicker and more abundant than the product of cells after 14 days. A faint band (PCR product) was detected in reactions missing the RT enzyme at day 14 and 21. Given that the *Runx2* primers lie on different exons, the presence of a PCR product at the expected size of amplification of mRNA in the negative reaction cannot be attributed to DNA contamination. Most likely there was cross contamination between PCR samples in the same run. In agreement with previous reports, a faint band of *Runx2* also appears in the lane representing cells grown in normal media at day 1 which indicate a low level of *Runx2* transcription. Figure-4A and -4B shows the PCR expression analysis of *Runx2* gene.

3-3-3 Levels of *Collagen1* mRNA expression under different media at different periods of times:

Dense bands were detected in lanes representing PCR *Collagen1* levels in cells grown in differentiation osteogenic media. The bands representing cells after 14 days are thicker than those produced by cells after 21 days. This may indicate that *Collagen1* expression is decreased with time during the periods of increased mineralization. Figure-4 accounts for the PCR analysis for *Collagen1* gene.

3-3-4 Levels of *Osteocalcin* mRNA expression under different media at different periods of times:

The dense abundant band was present in the lane representing cells grown in differentiation osteogenic media after 14 days from the start of the experiment indicating that *Osteocalcin* transcription was prominent at that time. The less prominent band was present in the lane representing cells under differentiation media after 21 days from the start of the experiment, indicating that the transcription of *Osteocalcin* decreased during the period between two and three weeks but still was transcribed at the end of the third week. A band for *Osteocalcin* was present also in the lane representing samples taken from cells cultured differentiation osteogenic media after 21 days, but the reverse transcriptase enzyme was not used in this sample during cDNA synthesis, so this band may be due to DNA contamination of the sample. Figure-4 accounts for the PCR analysis for *Osteocalcin* gene expression.

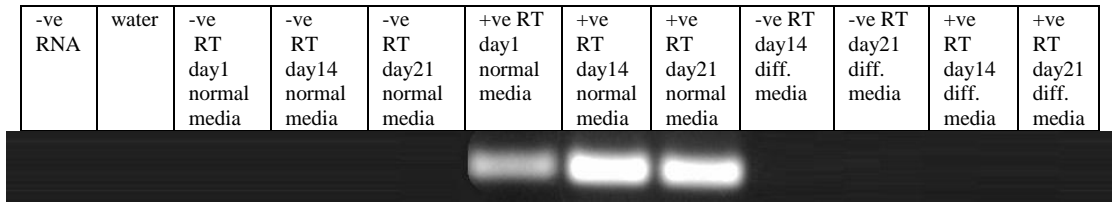
3-4 L929 cells cultured in osteogenic differentiation media display almost complete downregulation of *Cobra1* expression:

Transcription of *Cobra1* gene appears in the PCR analysis in the three lanes representing cells which were cultured in regular media that did not contain any osteogenic differentiation factors at all three-time points (day1, day14 and day21). No expression of *Cobra1* has been produced by cells cultured in osteogenic

differentiation media, indicating that *Cobra1* expression has been shut down during and after differentiation of osteoblasts at least between days 14 and day 21 from the beginning of the differentiation.

Figure (5) accounts for the PCR analysis for expression of *Cobra1* gene.

A)



B)

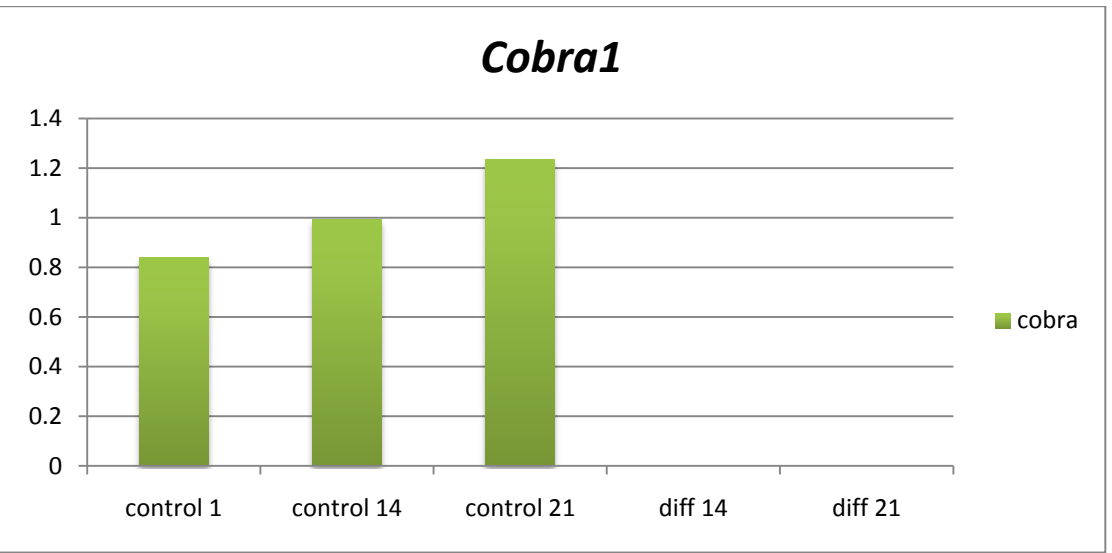


Figure-5: PCR reaction for gene expressions of *Cobra1*, *Cobra1* mRNA is detected in the un-induced but not the induced L929 cells. A) *Cobra1* expression in the cells grown in normal non differentiating media at day1, day14 and day21. The intensity of the bands increased with time until day 21. The size of the bands is 366 bp, that is indicative for *Cobra1* gene.

B) Graphical representation for the transcription levels of *Cobra1* by ImageJ, normalized to *Beta-actin*.

Chapter (4) Discussion:

Somatic fully differentiated cells could successfully convert to another type of mature somatic cells without the need to dedifferentiate the cells to obtain pluripotency before induction of differentiation to another cell type. This was apparent from the results of this research and previous findings as will be highlighted in the following sections.

It has been reported that the pluripotent stem cells retain their potency by maintaining the transcription of the main pluripotency genes such as Oct4, Sox2 and Nanog, and sustaining the suppression of the transcription of genes responsible for differentiation of pluripotent cells. Amleh and colleagues (**Amleh et al., 2009**) have demonstrated the involvement of COBRA1, which is an integral member of the NELF complex, in maintaining the pluripotency of mouse embryonic stem cells

In the current study, we induced the osteogenic differentiation of L929 using media containing a cocktail of Dexamethazone, Ascorbic acid and Beta-Glycerol phosphate and investigated the involvement of *Cobral* in the plasticity/potency of L929 cells. In a previous study, the observation of cellular changes after induction of differentiation was slower compared to the changes observed in the current study which will be detailed in the following sections; this may be due to increasing the concentration of Dexamethazone in the osteogenic differentiation media in the present study compared to the previous one (**Kasem, Tanom, 2011**).

4-1 Morphological changes:

The observation of cells in differentiation media after three and a half days from the onset of differentiation has shown apparent morphologic changes of those cells; the cells assumed triangular to cuboidal shapes (**Florian et al., 2009**) and contained prominent nuclei. These changes have continued as the observations after 1 week, 2 weeks and 3 weeks show. Areas of extracellular deposits could be seen around some cells after 1 week from differentiation, and these areas have increased by time to be more abundant after 2 and 3 weeks.

On the other hand, un-induced L929 cells maintained mostly the spindle-like shape at early time points and exhibited a noticeable increase in the round shaped cells with increasing time (**Michael et al., 2015**).

4-2 Staining of calcium deposits

In agreement with previously reported data, mineralized osteoblasts derived from L929 exhibit enhanced extracellular calcium deposits in response to an increased time of induction as depicted by bright red Alizarin red S staining (**Ji Eun et al., 2016**). However, in the current study, these changes were detected at an earlier time point than previously reported (**Kasem and Tanom., 2011**).

4-3 Effect of osteogenic media on transcription of *Runx2*:

There were no *Runx2* RNA detected in cells which were grown in regular media neither at day 14 or day 21. However, there was a faint band in the lane representing cells cultured in standard media at day 1 which may indicate a low level of *Runx2* transcription in fibroblasts (Masafumi et al., 2016). This discrepancy in *Runx2* gene expression levels could be due to the apparent overcrowding at days 14 and 21 or due to spontaneous formation of germline-like cells. It has been reported by (Ma et al., 2012) that L929 cells undergo spontaneous differentiation into germline-like cells within 7 days in regular culture medium. *Runx2* being the regulator for osteogenic differentiation (Aaron., 2013) is transcribed in cells that were culture in osteogenic differentiation media whether at day 14 or day 21 as indicated by the PCR analysis. It is noted that *Runx2* PCR product increased by time from 2 to 3 weeks, which may suggest the maturation of the differentiated cells into functional osteoblasts. These findings indicate the positive effect of the osteogenic differentiation molecules on the conversion of fibroblasts to osteoblasts.

4-4 Effect of osteogenic media on transcription of *Collagen1*:

In agreement with previous findings (Chan et al., 1991), (Gallivan et al., 1997).we have not detected *Collagen1* gene transcripts in cells cultured in the regular medium throughout the experiment period, whereas the induced L929 cells express *Collagen1* transcripts at days 14 and 21. The density of the band representing *Collagen1* transcription shows increased transcription at day 14 than that at day 21. This may indicate that the Collagen1 is required during early differentiation stages more than at the time when the mineralization machinery dominates.

4-5 Effect of osteogenic media on transcription of *Osteocalcin*:

Our results emphasize what has been already reported that *Osteocalcin* gene is required for osteogenic differentiation, thus expressed in osteoblasts (Lee NK et al., 2007) and is considered as a marker for osteoblasts detection. Here again, like the case with *Collagen1*, the transcription level of *Osteocalcin* is more prominent at day 14 than day 21 from the start of the experiment. However, it was noted that the difference in transcription pattern between day 14 and 21 is more evident in the case of *Osteocalcin*, where a thick band was present at day 14 while a relatively weak one was present at day 21. This may indicate that the role of Osteocalcin precedes the mineralization of osteoblastic matrix.

4-6 Effect of osteogenic media on transcription of *Cobra1*:

The transcription of *Cobra1* was apparent in the PCR reactions for cells grown in regular media at all times of the observation but not found in cells cultured in osteogenic differentiation media neither at day 14 or day 21. This may suggest a suppressive effect of the osteogenic media on the transcription of *Cobra1*. Alternatively, this may indicate that COBRA1, as an integral member of the NELF

complex, is required to maintain the plasticity of L929 and that for osteogenic differentiation to occur *Cobra1* levels are dampened. We do not know exactly how this happens, does the osteogenic molecules directly suppress the transcription of *Cobra1*? Or is the activated differentiation machinery induced by the osteogenic media involved in the downregulation of *Cobra1*? Further studies are needed to test the exact mechanism by which the transcription of *Cobra1* is suppressed.

4-7 Analysis of the transcription pattern of *Beta-actin*:

Beta-actin is considered as one of the house keeping genes which are transcribed normally and consistently in all living cells, and it was used in this experiment as a control for all reactions to test the validity of PCR reactions, to ensure the PCR reactions results for rest of products.

The *Beta-actin* was transcribed as expected in all reactions that include RNA and reverse transcriptase enzyme (+v RT) indicating that results obtained from the other PCR reactions are reliable.

Chapter (5): Conclusion

According to our findings, we can conclude that the use of osteogenic substances has successfully induced the mouse fibroblasts L929 to differentiate into mature and active osteoblasts.

It should be noted that in the present study the Dexamethazone used in the osteogenic differentiation media was of different concentration than previously recommended in the literature. This difference may have contributed to the early differentiation induction.

We also can conclude that the Collagen1 and Osteocalcin secretion has not increased with time and their level is less during the mineralization phase of osteoblasts.

Interestingly, the *Cobra1* expression was abolished on day 14 of induction. While the use of osteogenic differentiation media may have a suppressive effect on the *Cobra1* transcription, it appears that the presence of *Cobra1* transcription in the fibroblasts did not suppress the fibroblastic capacity to differentiate into osteoblasts. It is likely that COBRA1 is required to support the plasticity of L929 cells similar to the role it plays in maintaining the pluripotent state of the mouse embryonic stem cells.

This work may be considered as a foundation to understand the inhibitory effect of the differentiation media and conditions on COBRA1 transcription.

Future Recommendations

In this research, the timing for alizarin red S staining for calcium deposits and for RNA extraction from samples cultured in differentiation media (day 14 and day 21) were based on previous similar research strategies. In those studies-, the mineralization was proven not to occur before day 21 (**Kasem, Tanom, 2011**). Interestingly, in the current research, we observed that the morphological changes and the mineralization detection using the stain have occurred at earlier times than the previously reported. Therefore, we recommend monitoring the expression of osteogenic markers including the process of mineralization at an earlier stage; i.e., within a week of the onset of induction and to try different concentrations of Dexamethazone including the previously used one.

The analysis of *Cobra1* transcription was also too late. To better understand the role of COBRA1 in the maintenance of the L929 plasticity, the expression levels of *Cobra1* need to be examined within a few days of induction.

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