

# Effects of Functional Protein on Osteoblasts in Rat

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**Abstract**—To assess the effects of functional protein on osteoblast, Large quantity of high-purity osteoblasts had been cultivated successfully by adopting sequential enzyme digestion. The growth curve of osteoblasts was protracted by cell counting. Proliferation of osteoblasts was assessed by MTT colorimetry. The experimental results show the functional protein can enhance proliferation, the properties of adhesion and discuss the effect of osteopontin on osteoblast.

**Keywords**—functional protein; osteoblast; MTT

## I. INTRODUCTION

THERE are a number of functional proteins in bovine milk, such as casein, whey protein and so on. In recent, Some researchers find a new functional protein—osteopontin(OPN) which has relationships with bone remodeling. OPN was first described as a secreted 60KDa transformation-specific phosphoprotein [1]. OPN is a sialic acid-rich, highly phosphorylated glycoprotein that contains several cell adhesive domains, including an arginine-glycine-aspartic acid(RGD)-containing domain that interacts with cell surface integrins  $\alpha_v\beta_3$ ,  $\alpha_v\beta_1$ ,  $\alpha_5\beta_1$  integrin adhesive domain [2] and a cryptic, serine-valine-valine-tyrosine-glutamic acid-leucine-arginine(SVVYGLR)-containing domain that interacts with  $\alpha_9\beta_1$  following exposure by thrombin cleavage [3,4]. OPN from bovine milk has been demonstrated to contain a total of 28 phosphorylation sites(1 for threonine and 27 for serine residues) and three O-glycosylation sites [5]. OPN mediates important cellular functions by interacting via these domains with monocyte/macrophage derived cells reviewed by [6-8]. OPN has been described as an important regulator of these cells in respect of their adhesion, migration, differentiation, activation state, survival, and biomineralization [6]. It generally believed that OPN can induce Osteoblast (OB) differentiation, and promote the reconstruction of mineralized tissues. Noda [12] found that the process of bone remodeling in OPN Line and periosteal bone plate were significant higher. It is suggested that OPN may play an important role in adhesion processing and the cycle processing of bone metabolism. In addition, Wan [13] observed that the OPN expression within bone tissue was significantly reduced in the state of simulated

weightlessness, calcification function of OB is inhibited, while alkaline phosphatase (Alkaline Phosphatase, ALP) activity decreased, osteocalcin (Bone Gla Protein, BGP) secretion was also a significant decrease, it was one of the reasons that the expression of OPN declined may be bed-ridden and people who exercise reduced elavate the risk of osteoporosis. Therefore, the aim of the present work was to study OPN from bovine milk and the effect of OPN on OB.

## II. MATERIALS AND METHODS

### A. Materials

OPN was obtained from the Key Laboratory of Dairy Science[14].

### B. Methods

**OB Culture** : Referred to *in vitro* OB incubation method of Cuq P [15], 6 newborn Wister rats with 1d old were chosen, killed by breaking off neck, soaked in 75% alcohol for 10 min and then transferred to sterilized petri dish; the calvaria were taken out by cutting head skin, put into PBS to remove periosteum and peripheral connective tissue, digested 30min at 37°C by adding trypsinase and centrifuged 5min at 1000rpm, then the supernatant were abandoned. The precipitate was shaken and digested 2h at 37°C by adding collagenase type II, centrifuged 5 min at 1000 rpm, beaten upon repeatedly and washed 3 times with  $\alpha$ -MEM, and then above centrifugation was repeated.  $\alpha$ -MEM with 10% FBS was added to prepare cell suspension and adjust cell density to be  $10^5$ , the cell suspension was inoculated in culture bottle and incubated at 37°C in CO<sub>2</sub> incubator, and the medium was changed after 24h. Thereafter, the medium was changed once 2d, the cells were digested with trypsinase after spreading on culture bottle completely and subjected to subcultivation. The OB were purified before first subcultivation according to slightly modified method of Buckley[16]. Fibroblast adhered to vessel surface more easily than OB and its adhesion happened in 5min-10min, therefore, the prepared cell suspension was kept quiescently 10min, the supernatant was transferred to another culture bottle and kept quiescently again, the process was repeated 2 times, then purified OB can be gotten in last culture bottle. All cells used in this experiment were in third generation and were incubated 24h in  $\alpha$ -MEM free of serum before experiment.

#### 1.2.2 Cell Growth Curves

The third-generation OB were digested with 0.25% trypsinase and prepared into  $10^4$  cells/ml  $\alpha$ -MEM suspension, and then same amount of cells were exactly inoculated each hole of 24-hole plate. 3 holes were counted every day, the total cell count of each hole was determined and the mean value of

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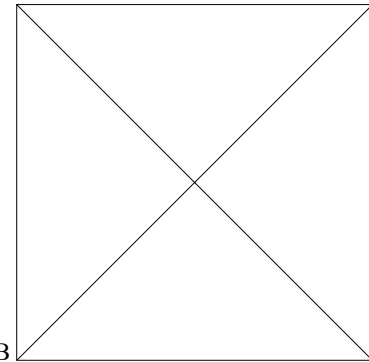
each group was gotten, which was lasted 8 days, lastly, the cell growth curve was drawn with Excel software.

**Cell Adhesion Assays :** Cell adhesion assays were performed in order to determine the ability of isolated OPN to promote OB adhesion. Polystyrene microwells (Corning-Costar, Corning, America) were coated with 50 $\mu$ l of bovine OPN purified as previously described at a concentration of 20 $\mu$ g/ml in phosphate buffered solution (PBS) overnight at 4 $^{\circ}$ C. After blocking with 10mg/ml BSA (bovine serum albumin) (Sigma) in PBS, wells were rinsed with PBS. OB were grown in  $\alpha$ -MEM and 10% fetal bovine serum. Cells were rinsed and resuspended in  $\alpha$ -MEM (1mg/ml BSA) at a density of  $3 \times 10^4$  cells/well. Cells were allowed to adhere for 30 min at 37 $^{\circ}$ C before submerging the entire plate in 1 liter of isotonic saline. All air bubbles were removed and nonadherent cells were removed by gently moving the plate in an up and down manner 20 times. Care was taken to prevent air from entering wells. With a single motion, the plate was inverted and fluid was removed to minimize variation in shear forces between wells. The plate was resubmerged in paraformaldehyde was added 5min. Cells were stained with 0.5% toluidine blue for 30min, rinsed, and solubilized with SDS to obtain an absorbance reading at 595nm which corresponds directly to the number of cells stained in each well [17-19]. In the experiment of anti-OPN inhibition, before the addition of cells, incubation (potency 11:6) 30min with anti-OPN [20].

**Methyl Thiazolyl Tetrazolium Assay (MTT) :** The passage cells digested by trypsinase after being culturing 24 in  $\alpha$ -MEM free of serum were inoculated in 96-hole plate with  $10^4$  cells/hole, 100 $\mu$ l  $\alpha$ -MEM free of serum and containing 2.5, 5, 10, 20 and 40 $\mu$ g/ml drug was added to each hole, respectively, each group were set 5 repeats. A bland control hole was set, into which 100 $\mu$ l  $\alpha$ -MEM free of cell and drug was added. At the same time, normal control hole was set, with 100 $\mu$ l/hole, which did not containing drug. The OB and their control groups were incubated at 37 $^{\circ}$ C with 5% CO<sub>2</sub> and saturated humidity. The proliferation ability of OB was determined after 1, 2, 3, 4 and 5d of incubation with MTT method. The MTT method follow as below: 20 $\mu$ l 5mg/ml MTT solution that had been filtered to remove bacteria was added to each hole, the culture medium was removed after being incubated 4 h at 37 $^{\circ}$ C with 5% CO<sub>2</sub> and saturated humidity, and then 100 $\mu$ l dimethyl sulfoxide was added to each hole, the 96-hole plate was shaken at low speed and room temperature to make purple crystal substance dissolve completely; absorption value of each hold was determined at 490 nm with optical densitometer for ELISA.

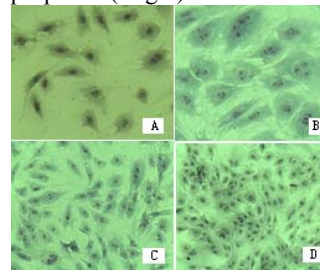
**Statistical analysis :** Repeat all the experiments more than 3 times, all data indicated by  $\bar{x} \pm s$  and analysed using SPSS 13.0 software. Comparison between the two groups using t test and multiple comparison using one-way analysis of variance (ANOVA). P < 0.05 was significant difference, P < 0.01 was extremely significant difference.

### III. RESULTS AND ANALYSIS



#### 2.1 Identification of OB

As for the effect of OPN on *in vitro* culture of rat OB, Large quantity of high-purity OB had been cultivated successfully by adopting sequential enzyme digestion, which laid the foundation for identification of OB as well as research on other properties( Fig.1).



- A. ALP staining 100 $\times$  (Third Generation, 1d)
- B. Geimsa staining 100 $\times$  (Third Generation, 1d)
- C. ALP staining 100 $\times$  (Third Generation, 3d)
- D. ALP staining 100 $\times$  (Third Generation, 3d)

Fig.1 Identification of osteoblast by Geimsa and ALP staining

#### 2.2 Cell Growth Curves

The growth curve of OB was protracted by cell counting, OB entered into rapid growth period the at 3rd day-7thd and reached peak at 6th day, and the cells proliferated exponentially(Fig.2).

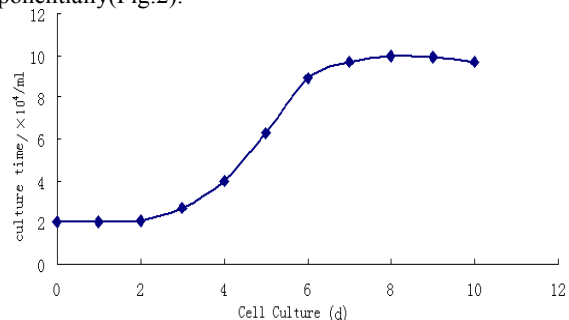


Fig.2 The growth curve of osteoblasts

#### 2.3 Cell proliferation assays

In cell proliferation experiment, when compared with control group, the OB proliferation of low-concentration OPN group (2.5 $\mu$ g/ml) was not notable ( $P > 0.05$ ), middle-concentration group (5.0 $\mu$ g/ml~10 $\mu$ g/ml) and high-concentration group (20 $\mu$ g/ml~40 $\mu$ g/ml) had remarkable proliferation ( $P < 0.05$ ,  $P < 0.01$ )(Table 1).

Tab 1 The effect of OPN on the growth of osteoblasts

(OD,  $\bar{x} \pm s$ , n=4)

Group µg/ml	MTT value at different time			
	1d	2d	3d	4d
Ref	0.133±0.006	0.171±0.003	0.191±0.002	0.222±0.004
OPN				
2.5	0.135±0.005	0.183±0.003*	0.193±0.006	0.257±0.005
5.0	0.168±0.005**	0.202±0.004*	0.293±0.020**	0.312±0.007*
10.0	0.248±0.020**	0.295±0.010**	0.340±0.020**	0.409±0.003**
20.0	0.268±0.010**	0.372±0.030**	0.393±0.006**	0.425±0.017**
40.0	0.332±0.003**	0.481±0.060**	0.470±0.005**	0.504±0.011**

\*Compared with the control group  $P < 0.05$ ; \*\*Compared with the control group  $P < 0.01$

#### 2.4 The properties of adhesion on osteoblast

Gene sequence analysis revealed that OPN contain RGD cell adhesion sequence which was the specific structure to cell with adherent activity. In this experiment, we wanted to isolate OPN from a natural source rather than work with recombinant OPN since posttranslational modifications such as phosphorylation or glycosylation may play a role in its biological activities. Moreover, some study showed that OPN promote cell adhesion which played a very important role in bone remodeling and bone mineralization. Therefore, Different concentrations of OPN on OB cell adhesion rate see Fig.3.

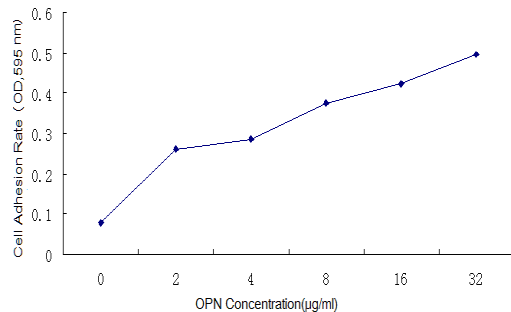


Fig.3 The effect of cell adhesion at different concentration of OPN

From these results, we can draw a conclusion that OPN can promote OB adhesion to extracellular matrix.

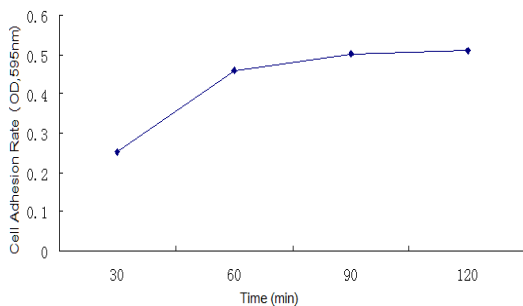


Fig.4 The effect of cell adhesion at different action time

From Fig.4, we can come to the conclusion, with the incubation time increasing, the rate of OB was upward trend. The adhesion rate reached maximum in 90min ~ 120min.

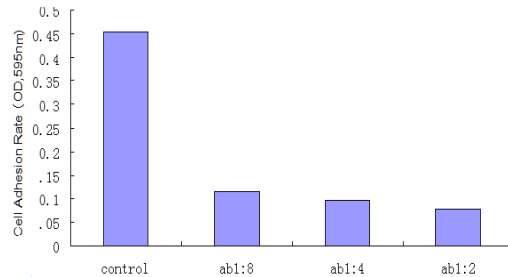


Fig.5 The inhibition effect of cell adhesion at anti-OPN antibody

The use of anti-OPN antibody-coated cell culture plate can inhibit OPN to promote adhesion function.

#### IV. CONCLUSIONS AND DISCUSSIONS

As for the effect of OPN on *in vitro* culture of rat OB. Large quantity of high-purity OB had been cultivated successfully by adopting sequential enzyme digestion, which laid the foundation for identification of OB as well as research on other properties. The growth curve of OB was protracted by cell counting, OB entered into rapid growth period at 3rd day-7th and reached peak at 6th day, and the cells proliferated exponentially. This was the same as literature [21]. OPN has been shown previously to promote adhesion through various integrins [22,23-25]. Although it is not clear how the adhesion-promoting activity of this preparation compares to ours. Our interest is in the biological activity of OPN and we wanted to minimize the exposure to potentially denaturing treatments. In cell proliferation experiment, when compared with control group, the OB proliferation of low-concentration OPN group was not notable, middle-concentration group and high-concentration group had remarkable proliferation. In ALP activity detection of OB, 10µg/ml and 20µg/ml OPN groups can improve the ALP activity at 3rd, 5th, 7th and 9th day, 40µg/ml OPN group can improve the ALP activity at all phases, it was not remarkable for 2.5µg/ml and 5.0µg/ml OPN groups to improve the ALP activity.

In conclusion, OPN can promote the proliferation and adhesion of OB. This provides basis for next step research about the functionality of osteopontin.

#### ACKNOWLEDGMENT

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#### REFERENCE

- [1] Senger DR, Wirth DF, Hynes RO (1979) Cell. 16:885-893
- [2] Liaw L, Skinner MP, Raines EW et al (1994) J. Clin. Invest. 95,713-724
- [3] Smith LL, Cheung HK, Ling LE et al (1996) J. Biol. Chem. 271,28485-28491
- [4] Yokosaki Y, Matsuura N, Sasaki T et al (1999) J. Biol. Chem. 274,36328-36334
- [5] Sørensen ES, Hørup P, Petersen TE (1993) Protein Science. 4,2040-2049
- [6] Giachelli CM and Steitz S (2000) Matrix Biology. 19,615-622
- [7] Denhardt DT, Noda M, O'Regan AW et al (2001) J. Clin. Invest. 107,1055-1061
- [8] Khan SA, Lopez-Chua CA, Zhang J et al (2002) J. Cell. Biochem. 85,728-736

- [9] Senger DR, Perruzzi CA, Papadopoulos A et al (1989) *Biochimica et Biophysica Acta*. 996,43-48
- [10] Rittling SR and Novck KE (1997) *Cell Growth and Differentiation*.8,1061-1069
- [11] Azuma N and Yamauchi K (1987) *J.Dairy Res.*54, 199-205
- [12] Noda M, Tsuji K, Nifuji A (2003) [J]. *Clin Calcium*.13,464-466
- [13] Wan YM, Ma YJ, Zhang XY(2005) [J]. *Acta Physiologica Sinica*. 57,384-388
- [14] Jie Sun, Guoyou Yin, Ning Liu.(2010).*Milk Science International*.65,131-134.
- [15] Cuq P, Marcelli C, Cousse H (1995) *C R Seances Soc Biol Fil*.189,303-12
- [16] Buckley MJ, Banes AJ, Jordan RD (1990) *J Oral Maxillofac Surg*.48,276-282; discussion 282-283
- [17] Lucy L, Michael PS, Elaine WR (1995) *J. Clin. Invest.* .2,713-724
- [18] Jun-Hyeog Jang and Ji-Hyun Kim (2005) *Biochnology Letters*.27,1767-1770
- [19] Kayla JB, Gerald AM, Martin SJ (1998) *J. Cell Sci.* 111,1165-1174
- [20] Liaw L, Almeida M, Hart CE (1994) [J]. *Circ Res* .74,214-224
- [21] Liu CJ, Liu JG, Yu TC et al (2007) *Chinese Journal of Gerontology*. 7,525-527.
- [22] Liaw L, Almeida M, Hart CE (1994) [J]. *Circ Res* 74,214-224
- [23] Sorensen S, Justesen SJ, Johnsen AH (2003) *Protein Expression and Purification* 30,238-245
- [24] Kon S, Maeda M, Segawa T et al (2000) *J. Cell. Biochem*.77,487-498.
- [25] Liu CJ, Liu JG, Yu TC et al (2007)*Chinese Journal of Gerontology* 7,525-527.