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Research Article

Expression Profiles of IGF-I, IGF-II, bFGF and TGF-β2 Growth Factors during Chicken Embryonic Development

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Abstract: Skeletal muscle development in avian embryos depends on the activation, proliferation, differentiation, and fusion of embryonic myoblasts. These processes as well as early embryonic development are mainly regulated by major growth factors such as IGF-I, IGF-II, TGF-β and FGFs. In order to determine their differential expression, total RNA was isolated from whole embryos on each of the embryonal days (E) 0 to 6, from the thoracic/abdominal half of the embryo at E 7 and E 8, and from pectoralis muscle tissues at E 9 to E 20. Growth factor cDNAs were synthesized by reverse-transcription polymerase chain reaction (RT-PCR). All growth factor messages except TGF-β2 were first detected as early as the blastula stage. IGF-I mRNA levels gradually declined on E 1 and remained lower through E 10. Levels then dramatically increased on E 11 (~ 3.5-fold) and remained high through E 13. On the other hand, overall the amount of IGF-II mRNA fluctuated from E 0 to E 20. Levels between E 0 and E 5 remained high and then declined (~ 3-fold) through E 16. TGF-β2 mRNA first appeared on E 3 and then levels remained high with slight fluctuations until E 18. bFGF mRNA levels increased (~ 2.5-fold) on E 3 and remained high with slight fluctuations until E 13. Because of the correlation of gene expression with the timing of major myogenic events, we suggest that IGF-I, bFGF and TGF-β2 are major regulators of embryonic skeletal muscle development in chickens.

Key Words: IGF-I, IGF-II, bFGF, TGF-β2, Chicken Embryos

Tavuk Embriyolarının Gelişim Sürecinde, IGF-I, IGF-II, bFGF, ve TGF-β2 Büyüme Faktörlerinin Expresyon Profili

Özet: Kanatlı embriyolarındaki çizgili kas gelişimi embriyonal myoblastların aktivasyonu, çoğalması, farklılaşması, ve füzyonuna bağlıdır. IGF-I, IGF-II, TGF-β ve FGF gibi büyüme faktörleri bu proseslerle birlikte daha erken olan embriyo gelişimini düzenler. Bu büyüme faktörlerinin ayrı ayrı expresyonunu belirlemek için, ilk 6 günde (E 0 ile E 6 arası) embriyonun tamamından, 7. ve 8. günde (E 7 ve E 8) göğüs ve karın kısmından, 9-20. günlerde ise (E 9 ile E 20 arası) sadece göğüs kaslarından RNA'lar elde edildi. Daha sonra, büyüme faktörlerine ait cDNA'lar, Reverse-Transcription Polymerase Chain Reaction (RT-PCR) tekniği kullanılarak sentez edildi. TGF-β2 haricindeki bütün büyüme faktörlerinin gen expresyonu en erken blastula aşamasında saptandı. IGF-I mRNA miktarı embriyonun birinci gününden itibaren azaldı ve 10'uncu güne kadar düşük seviyede kaldı. Diğer taraftan, genel IGF-II mRNA miktarı embriyonun 20'nci gününe kadar inişli çıkışlı seyretti. Şöyleki, 16'ncı embriyonik güne kadar 1-5'nci günler arasındaki seviyenin yaklaşık olarak 1/3'üne geriledi. TGF-β2 mRNA ilk olarak embriyonun 3'üncü gününde gözlendi ve miktarı küçük iniş çıkışlarla beraber embriyonun 18'inci gününe kadar yüksek kaldı. bFGF mRNA miktarı ise 3'üncü gününde arttı ve embriyonun 13'üncü gününe kadar küçük iniş çıkışlarla birlikte yüksek seyretti. Bu araştırmada gen expresyonu ile kas gelişimini düzenleyen mekanizmaların zamanlaması arasındaki uyuma paralel olarak, IGF-I, bFGF, TGF-β2'nin tavukların embriyonik çizgili kas gelişiminde önemli bir düzenleyici olabilecegi sonucuna varıldı.

Anahtar Sözcükler: IGF-I, IGF-II, bFGF, TGF-β2, Tavuk Embriyosu

Introduction

Although the effects of growth factors on cultured cells have been extensively studied, relatively little is known of their actual roles in embryos. One of the important steps in elucidating the functions of growth factors in embryos is the investigation of their expression pattern and relationship with developmental processes. Skeletal muscle is derived from somites migrating from the paraxial mesoderm into the adjacent region of the embryo (1). In chickens, myogenic cells start migrating from somites at approximately 2.5 days of incubation. The main myogenic period is spread over 10 days, beginning in the middle third of the embryonic period (2).

Insulin-like growth factors, IGF-I and –II, are the major requirements for the growth and development of chick embryos. They have been shown to stimulate

proliferation, differentiation and protein accretion of cells derived from avian embryos (3,4). Because of the absence of type-II IGF receptors, IGF-I and IGF-II have, unlike in mammals, similar activities in chicken embryos (4).

The transforming growth factor-β (TGF-β) family contains a large number of structurally related polypeptide growth factors that play a prominent role in the development and homeostasis of virtually all tissues in organisms. TGF-β1/β3 isoforms have been shown to be present in gastrulating chicken embryo and have functional effects on cell transformation, extracellular matrix deposition and cell proliferation (5).

Among the several extracellular factors that regulate myoblast proliferation and differentiation, the fibroblast growth factor (FGF) family plays a major role in skeletal muscle development. FGFs are required for the migration of muscle precursor cells in early chick embryonic development (6). The addition of FGFs has been shown to both delay the onset of myogenesis and stimulate proliferation of cultured mouse (7) and chicken (8) cells. Thus, the objective of the current study was to establish the ontogeny of IGF-I, IGF-II, TGF-β2 and bFGF gene expression with regard to embryonic and chicken skeletal muscle development.

Materials and Methods

Tissue collection

Fertilized eggs (Cobb X Cobb) were obtained from Wampler-Longacre (Moorefield, WV). Embryos and tissues were harvested in compliance with an approved West Virginia University Animal Care and Use Committee Protocol. All the embryos were isolated and washed free of yolk, albumen and extra-embryonic membranes by sterile nuclease-free water and were staged according to Hamburger and Hamilton (9). Whole embryos were collected on each of the embryonic days (E) 0 to 6 (stage 1 to stage 29, n = 6 per day). Thoracic/abdominal halves of the embryos between the lumbo-sacral level and the neck without the head were collected on each of E 7 and E 8 (stage 31 and stage 34, respectively, n = 6 per day). *Pectoralis* muscle was collected on each of E 9 to E 20 (n = 4 per day). All the tissue collections were performed at consistent times for each sampling day throughout the experimental period, starting on day 9, stage 35, and every 24 h until day 20, stage 45 (9).

RNA extraction

Total RNA was extracted from all of the tissues mentioned above using the Tri-Reagent (Sigma) modification of the guanidine isothiocyanate/phenol-chloroform method as described by Chomczynski and Sacci (10). The RNA concentration was estimated by absorbance at 260 nm in a Shimadzu spectrophotometer (Columbia, MD). Samples of RNA were stored at -80 ºC.

RT-PCR

Reverse transcription (RT) was performed by adding 2 µg of total RNA to 2 µg of Oligo dT primers and sterilized nuclease-free dd H_2O in a final volume of 15 µl. The samples were heated at 70 ºC for 5 min and then immediately cooled to 4 ºC over 2 min. RT buffer containing dNTPs (the final concentration of each was 10 mM), 25 units of RNase inhibitor, and 200 units of murine maloney leukemia virus reverse transcriptase (Promega, Madison, WI) were added to each sample. The sample, with a final volume of 40 µl, was incubated at 37 ºC for 1 h followed by a 5 min incubation at 95 ºC. For the PCR reaction, 2 µl of RT reaction mixture was added to 48 µl of solution containing 5 µl of Taq buffer, 1 µl of Taq DNA polymerase (Display Systems Biotech, Vista, CA), 1 µl of dNTPs (final concentration of each was 10 mM), 1 µl each of forward and reverse primers and 41 µl of sterile nuclease-free dd H_2O. The PCR reaction started with one cycle consisting of 94 ºC for 5 min, followed by an annealing step of 65 ºC (for bFGF) or 55 ºC (for IGF-I and β-actin) for 1 min and extension at 72 ºC for 1 min. The first cycle was followed by 30 cycles consisting of 30-s intervals of 94 ºC, followed by 65 ºC for bFGF or 55 ºC for IGF-I and β-actin, followed by 72 ºC. For IGF-II and TGF-β2, touchdown PCR was run. This program consisted of a 5 min, 94 °C denaturation step, followed by 5 cycles in which the initial annealing temperature of 72 °C for IGF-II and 65 °C for TGF-β2 was reduced by 1 °C per cycle, and then 30 cycles in which the annealing temperatures were 68 °C for IGF-II and 61 °C for TGF-β2. Denaturation, extention and annealing time were programmed as described above. A water (no cDNA) PCR reaction was used as a control for each gene and no contamination of reaction mixture components was found.

PCR primers

All PCR primers were obtained from Gibco BRL Inc. (Grand Island, NY). Primers for bFGF and TGF-β2 were designed on the basis of published sequences of chicken

bFGF and TGF-β2 (11,12). Primers for bFGF amplified a PCR product of 270 bp, which corresponded to bases 432-701 of the sequence, while primers for TGF-β2 amplified a PCR product of 269 bp, which corresponded to bases 6452-6722 of the sequence. The sequences of forward primers for bFGF and TGF-β2 were 5' GATCCGCACATCAAACTGC 3',

5' AGGAATGTGCAGGATAATT 3', while the reverse primers were

5' GATACGTTTCTGTCCAGGTCC 3', 5' ATTTTGGGTGTTTTGCCAA 3', respectively. Primers for IGF-I and IGF-II were designed on the basis of published sequences of chicken IGF-I and IGF-II (13,14). The sequences of forward primers for IGF-I and IGF-II were

5' GTATGTGGAGACAGAGGCTTC 3', 5' TGTGGAGGAGTGCTGCTTTC 3', while the reverse primers were 5' TTTGGCATATCAGTGTGGCGC 3',

5' GGGAGGTGGCGGAGAGGTCA 3', respectively. Forward and reverse primers amplified a PCR product of 200 bp for IGF-I, which corresponded to bases of 439-638, while primers for IGF-II amplified a 101 bp PCR product, which corresponded to bases of 44-145. Primers for β-actin were used to amplify a 285 bp product as previously published (15), as an internal standard for normalizing the level of amplification. The sequence of the forward primer was

5' TCATGAAGTGTGACGTTGACATCCGT 3', while the reverse primer was 5' CCTAGAAGCATTTGCGGTGCACGATG 3'.

The amplified PCR products for each gene were visualized on 1.5% agarose gels, stained with ethidium bromide. Products were analyzed by densitometry of stained gels, and data should therefore be considered on a semi-quantitative basis. The identity of all PCR products was confirmed by sequence analysis.

Results

β-Actin mRNA levels

β-Actin (house-keeping gene) was used for normalization of gene expression data and appeared to be consistently expressed throughout chicken embryonic development (Fig. 1).

IGF-I and IGF-II mRNA Levels

Expression of IGF-I and IGF-II genes were first seen during the blastoderm stage of chick embryo development. IGF-I mRNA levels gradually declined on E 1 and remained lower through E 10 (Fig. 2A). Levels then dramatically increased on E 11 (~ 3.5-fold) and remained high through E 13. IGF-I mRNA declined at E 14 (~ 2-fold) and plateaued through E 18. Levels then decreased prior to hatching. The highest and lowest IGF-I mRNA levels were seen in 11- and 6-day-old chick embryos, respectively. Overall the amount of IGF-II mRNA fluctuated from E 0 to E 20 (Fig. 2B). Levels between E 0 and E 5 remained high and then declined (~ 3-fold) through E 16. The highest and lowest IGF-II mRNA levels were seen in 3- and 15-day-old chick embryos, respectively.

TGF-β2 and bFGF mRNA Levels

TGF-β2 gene expression was first seen in 3-day-old chick embryos (Fig. 3A). The amount of TGF-β2 mRNAs remained high with slight fluctuations until E 18. Levels then declined prior to hatching (~ 4-fold). bFGF gene

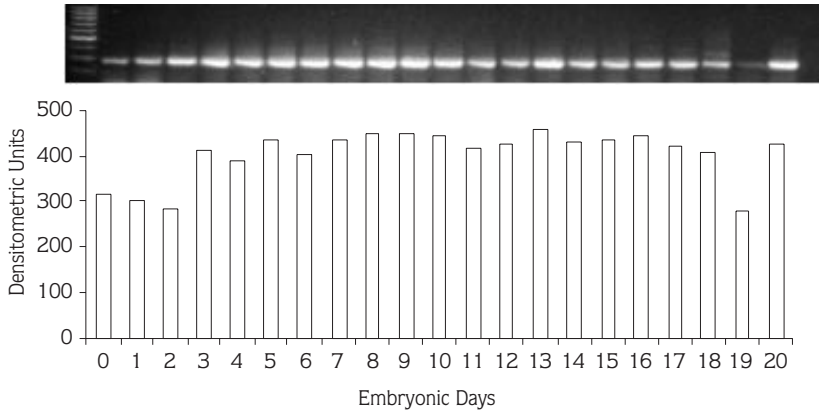

Figure 1. Steady-state levels β-actin mRNA in whole embryo, thoracic/abdominal half and skeletal muscle during chick embryo development (n = 6 or 4 per day, respectively). The bands for β-actin on the photograph were scanned by densitometer and the integration values (mean ± SD) are expressed in arbitrary densitometric units at each day.

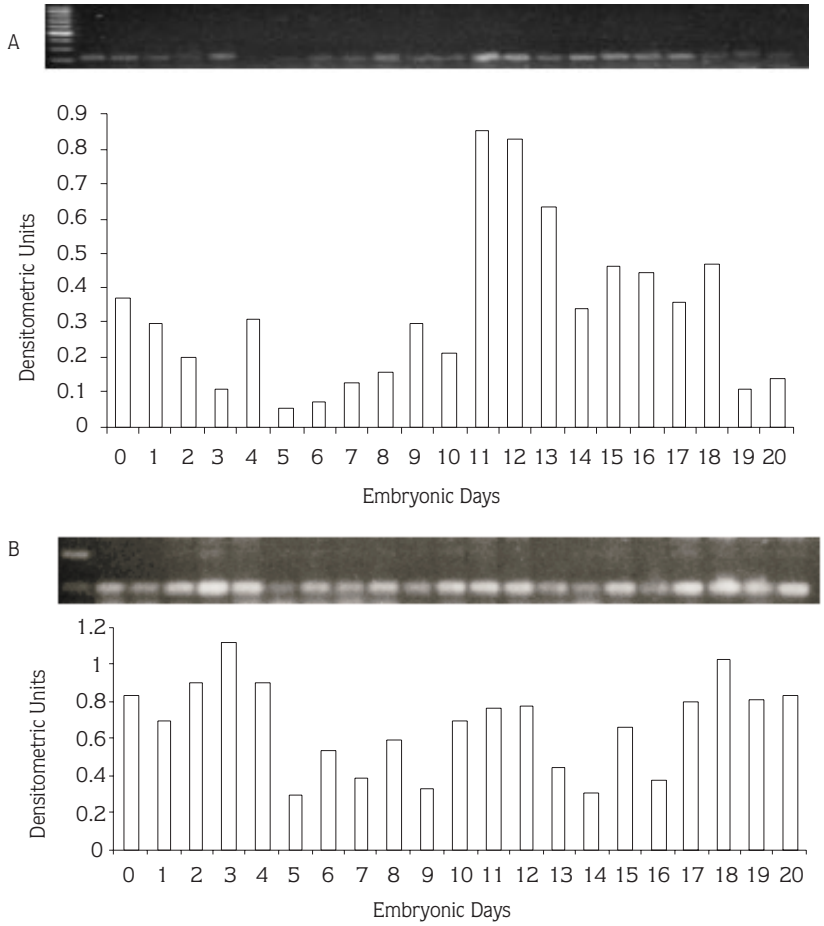

Figure 2. Steady-state levels of IGF-I (A) and IGF-II (B) mRNA in whole embryo, thoracic/abdominal half and skeletal muscle during chick embryo development (n = 6 or 4 per day, respectively). The bands for IGF-I and IGF-II on the photograph were scanned by densitometer and the integration values (mean ± SD), after normalization to β-actin, are expressed in arbitrary densitometric units at each day.

expression was first seen during the blastoderm stage of chick embryo development (Fig. 3B). The amount of bFGF mRNA remained low until E 8 with a sudden increase at E 3. Levels then increased (~ 2.5-fold) and plateaued until E 13. The highest and lowest bFGF mRNA levels were seen in 2- and 11-day-old chick embryos.

Discussion

Studies have shown that primary muscle formation occurs by E 7 and secondary muscle fiber formation occurs between E 7 and E 18 in chicken embryos (16-18). Approximately 80% of myofiber number is completed between E 7 and E 18 (major myoblast fusion starts at E 11).

Our results demonstrating that IGF-I mRNA accumulated to a peak level during mid-embryogenesis and then declined prior to hatching were in agreement with those of Kikuchi et al. (19). However, these authors were not able to detect IGF-I mRNA expression before E 3 by a nuclease protection assay as opposed to our findings of strong expression in the E 0 (blastoderm stage). Actions of IGF-II on vertebrata development, particularly in skeletal muscle growth, have not been fully established yet (20). For instance, post-natal growth of IGF-II null mice was similar to that of normal control mice (18), whereas transgenic mice overexpressing IGF-II grew more slowly than did control animals (21). The autocrine expression of IGF-II correlated with the rate of spontaneous differentiation of various muscle cell lines (22). In this respect, our findings of low IGF-II gene expression during mid-embryogenesis when most of the muscle fiber formation occurs were in disagreement with those in vitro findings.

The bioactivity of IGFs is modulated by IGF-binding proteins (IGFBPs) found in the extracellular fluids and serum of mammals. Even though mammals have six distinct classes of IGFBP, termed IGFBP-1 through

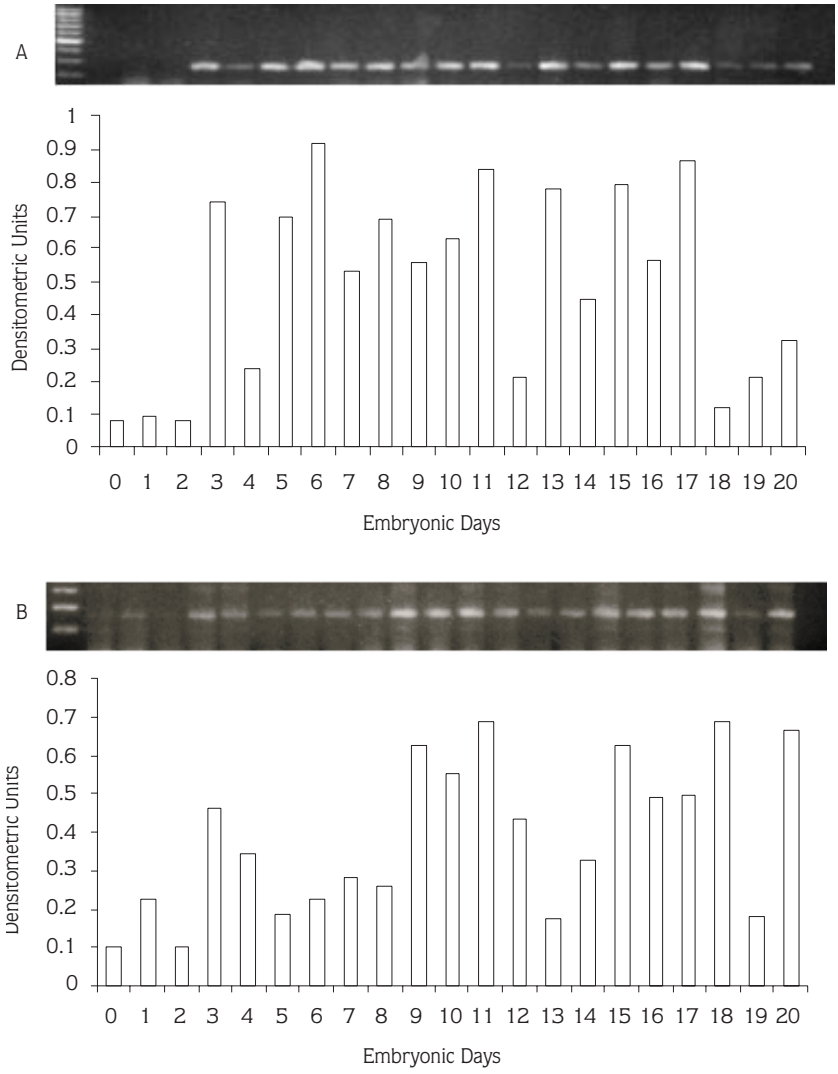

Figure 3. Steady-state levels of TGF-β2 (A) and bFGF (B) mRNA in whole embryo, thoracic/abdominal half and skeletal muscle during chick embryo development (n = 6 or 4 per day, respectively). The bands for TGF-β2 and bFGF on the photograph were scanned by densitometer and the integration values (mean ± SD), after normalization to β-actin, are expressed in arbitrary densitometric units at each day.

IGFBP-6, only IGFBP-2 (23) and IGFBP-5 (24) have been isolated from chickens. Therefore, to facilitate a better understanding of the regulatory roles of IGFs, it is important to establish the developmental expression pattern of these binding proteins in whole embryo and skeletal muscle tissues as well.

Sanders et al. (5) demonstrated that TGF-β1/β3 isoforms were expressed in gastrulating chick embryos and had functional effects on cell transformation, extracellular matrix deposition and cell proliferation. However, in the present study, TGF-β2 expression was not detected during early embryonic development. TGF-β2 mRNA levels increased in C2C12 myoblast cell cultures when the cells were allowed to differentiate in low-serum medium (25). Our results in which TGF-β2 mRNA expression increased during mid-embryogenesis when the majority of muscle differentiation takes place were in agreement with those in vitro findings. FGF is highly mitogenic for many muscle cell types and is also a potent inhibitor of myogenic differentiation in these cells (26). Additionally, Moore et al. (27) reported that FGFs and their receptor mRNAs in myoblast cell cultures were down-regulated during differentiation. However, in the current study, bFGF mRNA expressions were up-regulated during muscle differentiation (E 9 to E 18).

A complete understanding of the functions of growth factors and the activation of their gene expression in developing chicken embryos could be beneficial in food animal agriculture.

Acknowledgments

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References

1. Chevallier, A.: Role of the somitic mesoderm in the development of the thorax in bird embryos. II. Origin of thoracic and appendicular musculature. J. Emb. Exp. Morp. 1979; 49: 73-78.
2-. O'Neill, J.: Growth and differentiation during myogenesis in the chick embryo. Dev. Biol. 1987; 120: 465-480.
3. Girbau, M., Bassas, L., Alemany, J., dePablo F.: In situ autoradiography and ligand-dependent tyrosine kinase activity reveal insulin receptors and IGF-I receptors in prepancreatic chicken embryos. Proceedings of the National Academy of Sciences.1989; 86(15): 5868-5872.
4. Duclos, M.J., Wilkie, R.S., Goddard, C. Stimulation of DNA synthesis in chicken muscle satellite cells by insulin and insulin-like growth factors: evidence for exclusive mediation by a type-I insulin-like growth factor. J. Endocrinol. 1991; 128: 35-42.
5. Sanders, E.J., Hu, N., Wride, M.A.: Expression of TGF beta1/beta 3 during early chick embryo development. Anat. Rec. 1994; 238: 397-406.
6. Itoh, N., Mima, T., Mikawa, T.: Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle. Development 1996; 122: 291-300.
7. Linkhart, T.A., Clegg, C.H., Hauschka, S.D.: Control of mouse myoblast commitment to terminal differentiation by mitogens. J. Supramol. Struct. 1980; 14: 483-498.
8. Stern, H.M., Hauschka, S.D.: Neural tube and notocord promote in vitro myogenesis in single somite explants. Dev. Biol. 1995; 167(1): 87-103.
9. Hamburger, V., Hamilton, H.L.: A series of normal stages in the development of the chick embryo. J. Morphol. 1951; 88: 49-92.
10. Chomczynski, P., Sacci, N.: Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem.1987; 162: 156-159.
11. Borja, A.Z., Zeller, M.R., Meijers, C.: Expression of alternatively spliced bFGF coding exons and antisense mRNAs during chicken embryogenesis. Dev. Biol. 1993; 157: 110-118.
12. Burt, D.W., Paton, I.R.: Molecular cloning and primary structure of the chicken transforming growth factor-beta 2 gene. DNA and Cell Biol. 1991; 10: 723-734.
13. Kajimoto, Y., Rotwein, P.S.: Structure and expression of a chicken insulin-like growth factor I precursor. Mol. Endocrinol. 1989; 3 (12): 1907-1913.
14. Darling, D.C., Brickell, P.M.: Nucleotide sequence and genomic structure of the chicken insulin-like growth factor-II (IGF-II) coding region. Gen. Comp. Endocrinol. 1996; 102 (3): 283-287.
15. Yamamura, M., Uyemura, K., Deans, R.J., Weinberg, K., Rea, T.H., Bloom, B.R., Modlin, R.L.: Defining protective responses to pathogens: Cytokine profiles in lepropsy lesions. Science. 1991; 254: 277-279.
16. Feredette, B.J., Landmesser, L.T.: Relationship of primary and secondary myogenesis to fiber type development in embryonic chick development. Dev. Biol. 1991; 143: 1-18.
17. O'Neill, J.: Growth and differentiation during myogenesis in the chick embryo. Dev. Biol. 1987; 120: 465-480.
18. Stockdale, F.E., Nicovits, J.R.W., Christ, B.: Molecular and cellular biology of avian somite development. Dev. Dyn. 2000; 219(3): 304-321.
19. Kikuchi, K., Buonomo, F.C.C, Kajimato, Y., Rotwein, P.: Expression of insulin-like growth factor-I during chicken development. Endocrinology 1991; 128 (3): 1323-1328.
20. Ludwig, T., Eggeschwiler, J., Fisher, P., D'Ercole, A.J., Davenport, M.L., Efstratiadis, A.: Mouse mutants lacking the type 2 IGF receptor are rescued from perinetal lethality in IGF2 and IGF1r null background. Dev. Biol. 1996; 177: 517-535.
21. Mathews, L.S, Hammer, R.E., Behringer, R.R., D'Ercole, A.J., Bell, G.I., Brinster, R.L., Palmiter, R.D.: Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology 1988; 123 (6): 2827-2833.
22. Florini, J.R., Magri, K.A., Ewton, D.Z., James, P.L., Grindstaff, K., Rotwein, P.: "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. J. Biol. Chem. 1991; 266: 15917-15923.
23. Schoenle, T.J., Mazuruk, K., Waldbilling, R.J., Potts, J., Beebe, D.C., Chader, G.J., Rodriguez, I.R.: Cloning and characterization of a chick embryo cDNA and gene for IGFBP-2. J. Mol. Endocrinol. 1995; 15: 49-59.
24. Allender, S.V., Ehrenborg, E., Luhtman, H., Powell, D.R.: Conservation of IGFBP structure during evolution: Cloning of chicken insulin-like growth factor binding protein-5. Prog. Growth Factor Res. 1995; 6: 159-165.
25. Lafyatis, R., Lechleider, R., Roberts, A.B., Sporn, M.B.: Secretion and transcriptional regulation of the transforming growth factor-β3 gene during myogenesis. Mol. Cell. Biol. 1991; 11: 3795-3803.
26. Florini, J.R., Magri, K.A.: Assay of creatine kinase in microtiter plates using thio-NAD to allow monitoring at 405 nm. Analy. Biochem. 1989; 182: 399-404.
27. Moore, L.A., Arrizubieta, M.J., Tidyman, W.E., Herman, L.A., Bandman, E.: Analysis of the chicken fast myosin heavy chain family. Localization of isoform-specific antibody epitopes and regions of divergence. J. Mol. Biol. 1992; 225: 1143-1151.