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Differential proteome analysis of the cell differentiation regulated by BCC, CRH, CXCR4, GnRH, GPCR, IL1 signaling pathways in Chinese fire-bellied newt limb regeneration

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ABSTRACT

Following amputation, the newt has the remarkable ability to regenerate its limb, and this process involves dedifferentiation, proliferation and differentiation. To investigate the potential proteome during a dynamic network of Chinese fire-bellied newt limb regeneration (CNLR), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrum (MS) were applied to examine changes in the proteome that occurred at 11 time points after amputation. Meanwhile, several proteins were selected to validate their expression levels by Western blot. The results revealed that 1476 proteins had significantly changed as compared to the control group. Gene Ontology annotation and protein network analysis by Ingenuity Pathway Analysis 9.0 (IPA) software suggested that the differentially expressed proteins were involved in 33 kinds of physiological activities including signal transduction, cell proliferation, cell differentiation, *etc*. Among these proteins, 407 proteins participated in cell differentiation with 212 proteins in the differentiation of skin cell, myocyte, neurocyte, chondrocyte and osteocyte, and 37 proteins participated in signaling pathways of BCC, CRH, CXCR4, GnRH, GPCR and IL1 which regulated cell differentiation and redifferentiation. On the other hand, the signal transduction activity and cell differentiation activity were analyzed by IPA based on the changes in the expression of these proteins. The results showed that BCC, CRH, CXCR4, GnRH, GPCR and IL1 signaling pathways played an important role in regulating the differentiation of skin cell, myocyte, neurocyte, chondrocyte and osteocyte during CNLR.

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1. Introduction

Adult newt possesses excellent ability to regenerate appendages after amputation, which has been used as an important model for studies of limb regeneration (Nye et al., 2003). Newt Limb regeneration studies provide new insights into the probability for limb regeneration in adult mammals (See et al., 2013). Within a few hours after amputation, the wound is firstly sealed by wound epidermis, and then the dermal fibroblasts at the edges of the wound migrate under the wound epithelium. Meanwhile, osteocytes, myocytes, nerve cells, schwann cells and dermal fibroblasts dedifferentiate to become into mesenchymal-like stem cells, which accumulate under epidermis of the wound to form a blastema. Once formed, the accumulated blastema is enlarged to the bud stage by proliferation of these cells, which then differentiate to form the missing structures such as skin, muscle, nerve, blood vessels, cartilage, and bone (Vinarsky et al., 2005; Stevenson et al., 2006; Eguchi et al., 2011). Limb regenerative process in urodele amphibians can be divided into

Abbreviations: 2D-DIGE, two-dimensional differential gel electrophoresis; ANXA2, annexin A2; ATF2, activating transcription factor 2; BCC, basal cell carcinoma; CNLR, Chinese fire-bellied newt limb regeneration; CREB, cAMP response element binding protein; CRH, corticotropin releasing hormone; CXCR4, chemokine (C-X-C motif) receptor 4; DAG, diacylglycerol; EGR-1, early growth response protein 1; ELK-1, ETS domain-containing protein 1; ERK, extracellular regulated protein kinases; GNRH, gonadotropin-releasing hormone; GPCR, G-protein coupled receptor; GSK3β, glycogen synthase kinase 3β; IKK, inhibitor of nuclear factor kappa-B kinase; IL1, interleukin 1; IP3, inositol 1,4,5-triphosphate; ITPR1, inositol 1,4,5-trisphosphate receptor 1; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MS, mass spectrometry; NF-κB, nuclear factor κB; NOS1, nitric oxide synthase; p38MAPK, p38 mitogen activated protein kinases; PLCB, phospholipase C beta; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; SHH, sonic hedgehog; STAT3, signal transducer and activator of transcription 3

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four major stages: wound healing, dedifferentiation of adjacent cells to generate pluripotent cells; proliferation of pluripotent cells to form the regenerating blastema; differentiation and morphogenesis of the regenerating blastema (Jiang et al., 2014). Meantime, the differentiation process may be dependent on interaction between skin, muscle, nerve, blood vessels, cartilage, and bone cells. Proteomic analysis has previously been performed by several groups in both regeneration-competent and regeneration-incompetent limbs in both urodeles and frogs (Rao et al., 2009, 2014). Garza-Garcia et al. (2009) reported that Prod1, which is involved in the specification of newt limb proximodistal identity, was restricted to salamanders, and the absence of the Prod1 could be correlated with the lack of comparable limb-regenerative capability in other adult vertebrates. Looso et al. (2013) reported that almost 15,000 transcripts with protein coding potential resulted in the identification of 826 urodelian-specific proteins, which will be an indispensable resource for a better understanding of regenerative processes in newts at the molecular level. Therefore, an investigation of the potential proteome implicated in a heterogeneous mix of altered cell states during a dynamic network of newt limb regeneration, will make a significant sense to clinical medicine in the future.

Cell differentiation is a crucial stage of limb regeneration and is regulated by multiple signaling pathways. Although some studies support the notion that several signaling pathways such as CXCR4, SHH, Wnt, BCC, *etc*, may be important for limb development and regeneration (Hunger et al., 2012; Singh et al., 2012), their roles in cell differentiation during newt limb regeneration are incompletely defined. For example, in BCC signaling pathway, transcription factors TCF/LEF and Gli could be activated through Wnt pathway and SHH pathway (Anastas and Moon, 2013; Lin et al., 2014). In CRH and GnRH signaling pathways, transcription factors ATF2, CREB, ELK-1, EGR-1 and FOS could be activated through cAMP/PKA pathway and IP3/DAG pathway (Gur et al., 2001; Mayer et al., 2008; Sheng et al., 2012). In CXCR4 signaling pathway, transcription factors ELK-1, EGR-1, c-Jun and c-Fos could be activated through MEK/ERK pathway and PAK/JNK pathway (Takeuchi et al., 2012). In GPCR signaling pathway, transcription factors STAT3, CREB, ELK-1, NF-κB could be activated through JAK/STAT3 pathway, cAMP/PKA pathway and PI3K/Akt pathway (Luttrell, 2008; Kobayashi et al., 2010). In IL1 signaling pathway, transcription factor NF-κB, c-Jun and c-Fos could be activated through IKK/NF-κB pathway and p38 MAPK/JNK pathway (O'Neill, 2008; Virtue et al., 2012). These activated transcription factors could regulate cell differentiation. In addition, Singh et al. (2012) have reported that a coordinated interaction between signaling pathways may be necessary for cell differentiation in limb regeneration.

In order to illustrate the mechanisms of cell differentiation during Chinese fire-bellied newt limb regeneration (CNLR) at the proteome level, we detected protein expression profiles during CNLR using high resolution two-dimensional differential gel electrophoresis (2D-DIGE) and mass spectrometry (MS), and studied the correlation of the cell differentiation activity and the signal transduction activity by IPA software. The results suggested that BCC, CRH, CXCR4, GnRH, GPCR and IL1 signaling pathways mediated the differentiation of skin cell, myocyte, neurocyte, chondrocyte and osteocyte during CNLR.

2. Material and methods

2.1. Model preparation of Chinese fire-bellied newt limb regeneration

Chinese fire-bellied newts (*Cynops orientalis*) were purchased in Jigong Mountain of Xinyang in Henan province. A total of 165 well-grown adult Chinese fire-bellied newts were randomly divided into 10 experimental groups and one control group with 15 Chinese fire-bellied newts per group. The forelimbs of Chinese fire-bellied newts were amputated in distal portion of stylopod (Humerus) on the right side. The distal tissue with 2 mm was removed at the amputation site and was served as control group. The regenerating tissues with 2 mm in each group were collected at 2 h, 8 h, 1 d, 2 d, 4 d, 5 d, 6 d, 12 d, 20 d and 28 d after amputation, respectively. The samples were stored in liquid nitrogen for further use. All experiments were performed in strict accordance with the Animal Protection Law of China.

2.2. Histology

For histology, the control limb tissues were fixed in Bouin's solution for 48 h. The fixed tissues were washed in 50% and 70% alcohol in order, and then were dehydrated in a graded series of alcohol, followed by xylene. After that, they were infiltrated overnight with paraffin. The tissues were then sectioned at 10 μm. Sections were stained with hematoxylin and eosin, and photographed on a Leica microscope (LEICA, Germany).

2.3. Proteins extraction and fluorescent dye labeling

Proteins extraction was performed according to the method previously described (Yang et al., 2013). The regenerating tissues were placed in liquid nitrogen to grind into fine powder, and then were suspended in lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5). Next, the suspension was vortex-mixed at 4 °C for 1 h, and then was centrifuged at 20,000*g* for 1 h. The supernatant was collected and stored at −80 °C for further use. The concentration of total proteins was determined using a 2D Quantification kit (GE Healthcare, USA).

The fluorescent labeling of proteins was performed following the standard protocol for minimal labeling according to Ettan DIGE User Manual (GE Healthcare, USA). Briefly, 50 μg proteins from control group were labeled with Cy3, and 50 μg proteins from each experimental group were respectively labeled with Cy5. On the other hand, 25 μg proteins from control group and 25 μg proteins from 10 experimental groups mentioned above were pooled together, labeled with Cy2 as internal reference proteins. The labeling reactions were performed on ice in the dark for 30 min, then quenched by addition of 1 μL of 10 mM lysine for 10 min and then stored at −80 °C for use (Di Luca et al., 2013).

2.4. 2D-DIGE and imaging analysis

The two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed according to the operating manual (GE Healthcare, USA). In brief, 50 μg of Cy2-, Cy3-, Cy5-labeled samples were pooled together and then dissolved in 450 μL rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 18 mM DTT, 2% IPG buffer 3–10). Then IPG gel strip (pH 3–10 NL, 24 cm) were rehydrated in the rehydration buffer for 13 h at 20 °C. The isoelectric focusing (IEF) was performed in Ettan IPGphor (GE Healthcare, USA) following the conditions: ramped to 250 V in 1 h, held at 1000 V for 3 h, ramped to 10,000 V in 3 h, and held at 10,000 V for 8 h. After IEF, each gel strip was equilibrated for 15 min in 15 mL equilibration buffer (50 mM TrisHCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT) and subsequently alkylated for 15 min in alkylation equilibration buffer (50 mM TrisHCl pH 8.8, 6 M Urea, 30% v/v glycerol, 2% SDS, 2.5% iodoacetamide). Subsequently, the proteins in gel strip were separated by SDS-PAGE, which was performed in Ettan DALT Six electrophoresis device (GE Healthcare, USA) under the following conditions: held at 50 V for 2 h, then at 120 V for 8 h.

After 2D-DIGE, all the gels were scanned at 100 μm resolution using a Typhoon FLA 9500 scanner (GE Healthcare, USA) according

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