Microarray Cluster Analysis of Irradiated Growth Plate Zones Following Laser Microdissection

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Introduction: Children who receive radiotherapy for bone or soft-tissue sarcomas in areas close to a growth plate are at high risk for developing growth arrest. Some capacity for growth plate recovery after irradiation appears to exist, based on the variability of clinical outcomes following irradiation of the growth plate and on evidence from our own work in the weanling Sprague-Dawley rat model. However, the mechanisms of post-radiation growth plate recovery are poorly understood. In order to develop potential selective radiorecovery agents for clinical use during radiotherapy treatment of pediatric solid tumors, our recent work has focused on obtaining a better understanding of early growth plate recovery. It is postulated that identification of key early upregulated genes may provide an understanding of the mechanism of this recovery and lend itself to the development of novel radiorecovery agents. Our hypothesis was that differential upregulation of specific gene expression exists between irradiated and non-irradiated reserve zone (RZ), proliferative zone (PZ), perichondrium (PC) and hypertrophic zone (HZ) chondrocytes and that some factors potentially vital to growth plate recovery would follow a pattern of early upregulation followed by a decrease in expression.

Materials and Methods: Six 5 week male SD rats underwent fractionalized irradiation to the right tibiae over 5 days totaling 17.5 Gy. The left side tibiae served as non-irradiated controls. At 7, 11 and 16 days following the first radiotherapy fraction, animals were euthanized and their left and right proximal tibial growth plates were collected. Total RNA was isolated from the RZ, PZ, HZ, and PC chondrocytes after separation by laser microdissection. RNA from each rat tibial growth plate zone and time point was labeled and amplified individually, pooled into two samples representative of three animals each, and applied to either of two RAE 230 2.0 chips.

To identify differentially expressed genes in the right irradiated tibia compared to the left non-irradiated tibia and to compare the 7, 11 and 16 day irradiated tibia, Genespring GX was used. Meaningful differential expression was determined at the 99.9% confidence interval, having a minimum expression of 10x up or down and cutoff of upregulation at seven days. Additionally genes were clustered by self-organizing map (SOM) and pathway analysis. Real-time RT-PCR was performed with SYBER green to confirm selected microarray findings.

Results: A 3x2 SOM was performed to cluster RZ genes, and three of the six clusters showed progressive upregulation from 7 to 11 to 16 days. Two clusters (1,2) and (1,3) fit the pattern of significant upregulation on day 7 with expression decreasing on days 11 and 16. On pathway analysis of 23 RZ genes meeting the differential expression level filters, enrichment was seen for 16 GO ontology pathways. Of these, none specifically involved bone, cartilage, matrix and/or skeletal development (BCMSD) and none had a minimum of 5 probe sets from our data set involved.

Since 10 of 12 PZ clusters in the 4x3 SOM followed the hypothesized pattern of early upregulation, pathway analysis was completed on the entire group of 244 genes. This showed 103 enriched pathways with a minimum of 2 probe sets and a minimum fold enrichment of 5. Of these 16 pathways (16%) were involved in BCMSD. With a 5 probe set minimum, the PZ showed 12 BCMSD pathways and 35 other pathways involved.

For the PC genes, all clusters from the 3x2 SOM showed the trend of hypothesized importance. Therefore, pathway analysis of the group of 18 PC genes passing the differential expression level filter was done. This showed enrichment of 52 pathways with a minimum of 2 probe sets per pathway from our data set, of which 4 pathways (8%) were involved in BCMSD. Using a 5 probe set minimum, the PC showed no BCMSD pathways but 15 other pathways involved.

For the HZ genes, all clusters from the 4x3 SOM showed the temporal trend of hypothesized importance. Therefore pathway analysis of the entire group of 245 HZ genes was done. 201 pathways showed enrichment with a minimum 2 probe sets per pathway, of which 19 pathways (9%) were involved in BCMSD. Using the 5 probe set minimum, the HZ showed the same 12 BCMSD pathways as the PZ (Table I) and 61 others involved.

Discussion: Based on the current work, we can confirm our hypothesis that differential upregulation of specific gene expression exists between irradiated and nonirradiated RZ, PZ, PC and HZ chondrocytes and that a plethora of factors potentially vital to growth plate recovery do follow a pattern of early upregulation followed by a decrease in expression. In isolating specific zones that may be the most important in stimulating early regeneration of the growth plate after irradiation (using our hypothesized temporal pattern of importance), both the PZ and HZ showed involvement of 12 BCMSD pathways using the most stringent criteria. Neither the RZ nor PC had any pathways that fit these criteria. Further work is needed on the specific role of these limited pathways in stimulating regenerative clones.

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<th>GO ID</th>
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Table 1. Enriched pathways (independent of time course) related to BCMSD comprised of 5 or more probe sets from our data and their corresponding zones

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