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Early Gene Expression in Salivary Gland After Isoproterenol Treatment

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ABSTRACT

Isoproterenol, a β-adrenergic agonist, has been shown to induce salivary gland hyperplasia. However, the mechanism involved in this pharmacological phenomenon is not well understood. To gain a better understanding of the underlying changes, including genes, networks and pathways altered by isoproterenol, microarray-based gene expression analysis was conducted on rat parotid glands at 10, 30, and 60 min after isoproterenol injection. After isoproterenol treatment, the number of differentially expressed genes was increased in a time-dependent manner. Pathway analysis showed that cell hyperplasia, $p38^{MAPK}$, and IGF-1 were the most altered function, network and pathway, respectively. The balanced regulation of up- and down-expression of genes related to cell proliferation/survival may provide a better understanding of the mechanism of isoproterenol-induced parotid gland enlargement without tumor transformation. J. Cell. Biochem. 116: 431–437, 2015.

KEY WORDS: parotid gland enlargement; β-adrenergic receptor; gene ontology and pathway analysis; MAPK pathway

Saliva is a major defense mechanism for preventing oral infection. Salivary gland disorders, such as Sjögren's syndrome, and radiation damage to the salivary glands during head and neck cancer therapy, cause hyposalivation which results in rampant and severe oral diseases (e.g., caries and *Candida* infection) as well as a compromised quality of life [Ng and Bowman, 2010]. Unfortunately, to date, there is no treatment that can restore/repair damaged salivary glands. Development of strategies to preserve or regain

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salivary gland function is a great research interest for management of patients with salivary diseases.

It has been known for more than 50 years that the activation of the β-adrenergic receptor with isoproterenol induces rodent salivary gland hyperplasia/hypertrophy and acute stimulation of salivary protein secretion [Selye et al., 1961; Buchner and Sreebny, 1972]. The effect of isoproterenol on salivary gland enlargement is interesting because this is one of the few conditions where there is tissue regrowth without neoplastic transformation. To date, isoproterenol-induced salivary gland enlargement has been extensively studied at the morphological and pharmacological level. In contrast, there has been little progress in understanding the underlying molecular mechanisms by which isoproterenol affects salivary gland proliferation and differentiation.

In this study, we have evaluated early gene expression profiles of rat parotid gland in response to isoproterenol treatment using a high throughput rat whole genome microarray and performed interaction network and pathway analyses. We have tentatively identified potential early target genes and pathways regulated by isoproterenol treatment.

MATERIALS AND METHODS

ANIMAL CARE AND ISOPROTERENOL TREATMENT

Male Sprague–Dawley rats (2–3 months old) were purchased from Harlan Laboratories (Indianapolis, IN) and fed Teklad (Harlan Laboratories) mouse/rat diet ad libitum in an accredited facility using a 12 h day/night cycle. The rats were injected intra-peritoneally with isoproterenol (20 μg/g body weight) or normal saline (control). The rats were euthanized at the indicated times and the harvested parotid glands stored at −80°C until analyzed. All procedures involving animals were performed according to a protocol approved by the Institutional Animal Care and Use Committee, Audie L. Murphy Division, South Texas Veterans Health Care System.

RNA AMPLIFICATION

Total RNA was isolated from tissues using TRI Reagent (Molecular Research Center, Cincinnati, OH) and then treated with RNase-free DNase I (Applied Biosystems, Foster City, CA). cDNA synthesis was performed by reverse transcription (Invitrogen, Carlsbad, CA). For synthesis of cRNA, an Illumina TotalPrep RNA Amplification Kit (Life Technologies, Foster City, CA) was used to amplify 500 ng of total RNA. The quality of the cRNA was assessed using an Agilent RNA 6000 Nano Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The quantity of cRNA was measured (NanoDrop ND-1000 spectrophotometer, Thermo Scientific, Wilmington, DE) and 750 ng was used for analysis.

WHOLE GENOME ANALYSIS

Whole genome expression analysis was performed using Illumina RatRef-12 Expression BeadArrays according to the manufacturer's protocol (Illumina, San Diego, CA). Each microarray provides genome-wide transcriptional coverage of well characterized genes and gene candidates selected from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database (Release 16). The RatRef-12 BeadChip contains twelve arrays, each with >22,000 probes, allowing the processing of 12 samples in parallel.

The microarrays were scanned using an Illumina BeadArray Reader and SentrixScan software (Illumina). To generate gene expression data for analysis BeadStudio software (Illumina) was used. BeadStudio reports quality of performance based on built-in experimental controls.

DATA PROCESSING AND ANALYSIS

Quantile normalization was performed on the log2-transformed expression value (MATLAB/Bioinformatics Toolbox, MathWorks, Natick, MA). To determine differentially expressed genes, we compared rat salivary gland microarray data after isoproterenol injection (10, 30, and 60 min time points) with controls (0 min time point). The hierarchical clustering (heatmap) of genes with differential expression fold-change larger than 2.0 (log2-transformed) in at least one time point (10, 30, or 60 min vs. control) was generated with Pearson correlation coefficient and average linkage.

STATISTICAL ANALYSIS

We first determined intrinsic gene expression variation (thus the selection of twofold-change) of microarray assays by assuming a log2-transformed expression ratio to be normally distributed with zero mean, if we chose two replicated samples at time 0 (replicated arrays). For a given microarray, the probability of a gene with a log2-ratio >1.0 (twofold change) is $p=\Phi_{\mu,\sigma}(x>1)$, where $\Phi_{\mu,\sigma}(\cdot)$ is the normal distribution function with mean $\mu=0$ and standard deviation σ determined from the array. By using the binomial distribution, $P=\sum_{k=n}^{N}\binom{N}{k}p^k(1-p)^{N-k}$ where $N=22{,}517$ genes covered by the array, we determined that it is unlikely ($P\sim 0$) to observe 1 or more differentially expressed genes (DEGs) with a log2 fold-change of 1.0.

Gene ontology (GO) enrichments were performed at each time point by using DAVID (The Database for Annotation, Visualization and Integrated Discovery; [Huang et al., 2009]). Only enriched ($P<0.01$) GO terms in biological process category were selected to construct the pathway enrichment heatmap. We used the average gene expression values over all genes within the selected GO terms to represent the activation (red) or suppression (blue) of the function in the heatmap. In addition, we used Ingenuity Pathway Analysis software (IPA, Ingenuity System Inc., Redwood City, CA) for function, interaction network and pathway analysis for DEGs derived at each time point.

QUANTITATIVE RT-PCR

Total RNA samples were treated with RNase-free DNase I (Applied Biosystems). cDNA synthesis was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. qRT-PCR was performed on an ABI 7500 Sequence Detection System (Applied Biosystems) and subjected to RT-PCR and DynAmoTM SYBR® Green qPCR (Applied Biosystems). The oligonucleotide primer sequences used were: c-fos pair "forward" 5′- GGGAGTGGTGAAGACCA-TGT-3′; "reverse" 5′-CGCTTGGAGCGTATCTGTC-3′; C4 "pair forward" 5′-CAG-GAGGTGAAAGGCTCAGT-3′; "reverse" 5′-CAAAGAGGCCACAAC-TCCTC-3′; GADD45B "forward" 5′-CGGCCAAACTGATGAATGT-3; "reverse" 5′-GGGTGAAGTGAATTTGCAGAG-3′; GADPH "forward"

5′-TA-TGACTCTACCCACGGCAAGT-3; "reverse" 5′-ATACTCAGC-ACCAGCATCACC-3′. Gene expression was then normalized using the housekeeping gene GAPDH.

RESULTS

To examine the biological function of each DEG at the different time points, we performed DAVID to explore gene ontology enrichment. The results are shown in Figure 1C. At 10 min, extracellular matrix (ECM) organization (e.g., collagen fibril organization, ECM receptor interaction and focal adhesion; $P \sim 10^{-5}$ to 10^{-12} respectively, Benjamini-Hochberg FDR adjusted), immune/inflammation response ($P \sim 10^{-4}$), response to stimulus ($P \sim 10^{-2}$ to 10^{-4}), and regulation of cell growth ($P \sim 10^{-2}$ to 10^{-4}) were found to be "enriched." Among the *down regulated genes*, ECM related genes (*Col1A1*, *Col1A2*, *Col3A1*, *Fn1*, and *Ccdc80*) were highly down-regulated (>3 fold vs. time 0). The immune/inflammatory response genes (*C1qb*, *Serping1*, *C1s*, and *Mmp14*) were also down regulated (>2 fold). In contrast, the *up-regulated genes* included the response to stimulus and cell-growth process genes (*Btg2*, *Jun b*, *Wisp2*, and *Ccn5*). Strikingly, *Rasd1*, a Ras related protein known to function in suppressing cell growth, was over-expressed at 3.5 fold above untreated cells. Other genes known to be associated with cell growth (*Igfbp6* and *Igfbp7*; insulin-like growth factor binding protein 6 and 7) were suppressed at 10 min.

Fewer functions were dysregulated (both down- and up-regulated) at 30 min, which perhaps is due to the fact that some of the dysregulated genes start to recover by that time, while other genes are continuing to respond at 60 min (e.g., genes related to stimulus-response, transcription, and DNA binding).

Ingenuity pathway analysis (IPA) was used to identify function, network and pathway interactions among the DEGs. Figure 2A displays in graphical form the "enriched" biological functions and potential disease associations represented by the DEGs at 10, 30, and 60 min after isoproterenol injection. The biological function analysis indicates that altered expression was mostly associated with cell morphology ($P < 10^{-17}$ at 10 min), cell growth and proliferation ($P < 10^{-11}$ at 30 and 60 min) and cell death and survival ($P < 10^{-8}$ for all 3 time points) (Fig. 2A, Supplement Table S1). Genes associated with hyperplasia were also examined since isoproterenol induces salivary gland hyperplasia ($P < 10^{-3}$ at 10 min; $<10^{-5}$ at 30 min; not enriched at 60 min). At 30 min, genes for hyperplasia were either up-regulated (*c-fos*, *Btg2*, *Gadd45B*)or down-regulated (*Per2*, *Mmp14*, and *Ptprb*). Interestingly, at the earlier time point (10 min), some genes were clearly displaying differential expression (*Cyr61 and Junb* [over-expression] and *Mmp14* and *Per2* [under-expression]). A DEG uniquely up-regulated at 10 min was hydroxysteroid (11-β) dehydrogenase 1 (*Hsd11b1*).

Canonical pathway analysis showed that the complement system was the top pathway at 10 min, while the intrinsic prothrombin activation pathway was found at 30 min and the LPS/IL-1 mediated Inhibition of RXR function was at 60 min. Among the genes involved in the complement pathway, *C1q*, *C1r*, *C1s*, and *C4* were consistently reduced by isoproterenol. Interestingly, the Insulin-like growth factor 1 (IGF-1) signaling pathway was found to be significantly enriched at the 10 and 30 min ($P = 0.001$ and 0.00015, respectively) time points (Fig. 2B and Supplement Table S2). Close examination revealed that multiple IGF binding proteins (*Igfbp3*, *6*, and *7*) were suppressed at 10 min by isoproterenol treatment. Only *Cyr61*, a highly conserved cysteine-rich protein without IGFBP function [Grotendorst et al., 2001], was over-expressed at all time-points.

IPA for DEGs at 10 min, revealed that Cell Morphology, Cell Movement, and Cancer were the top three networks affected by isoproterenol treatment (Supplement Table S3). The first network contains mostly genes from the complement system (Fig. 2B). The second network contains genes related to IGF-1 signaling (Fig. 2B) and the third network is related to $p38^{MAPK}$ (with an enrichment score of 24) signaling (Fig. 2C). Genes involved in $p38^{MAPK}$ signaling, including *Jun B*, as well as *Gadd45γ* and *Gadd45B* (not shown in Fig. 2C), were significantly increased after isoproterenol exposure. These genes act as downstream or upstream factors for $p38^{MAPK}$ signaling in regulating cell proliferation.

To confirm the reliability of the microarray analyses, real-time PCR was performed on three altered genes (*c-fos*, *Gadd45b*, and *C4*). The results showed changes that paralleled those found with the microarray assessment (Fig. 3). The early gene *c-fos* was increased at both 30 and 60 min, whereas *C4* expression was immediately reduced at 10 min and remained low at 30 and 60 min post isoproterenol injection. For *Gadd45b*, both microarray and real time PCR showed an elevation at 30 and 60 min with some fold discrepancy at 60 min. When this discrepancy was re-evaluated in a separate microarray analysis, the ratio was found to be consistent with the real-time PCR results at 60 min (data not shown).

DISCUSSION

A number of studies have shown that isoproterenol induces salivary gland hyperplasia and hypertrophy in rats [Matsuura and Suzuki, 1997]. This pharmacologic effect is different from uncontrolled tumor growth since it is a self-limiting proliferative response. In earlier studies, we focused on identifying individual pathways associated with isoproterenol stimulation in salivary gland cells [Yeh et al., 2012]. To date, a system biology approach of salivary gland response to isoproterenol has been very limited [Ten Hagen et al., 2002]. In the current study, we have used high throughput microarray and Ingenuity Pathway analyses to show that more than 1,000 gene changes occur within 60 min in rat parotid gland in response to β-adrenergic stimulation (Table I). The affected genes are associated with cell growth/survival function as well as $p38^{MAPK}$, IGF-1 signaling, and complement pathways.

The β-adrenergic receptor plays a number of roles in salivary gland physiology, including protein secretion, secretory protein production and postnatal development and growth [Barka, 1990]. In our study, isoproterenol induced many early gene changes that were identified by DAVID and IPA. Changes in cell morphology and immunology/inflammation may reflect some of these non-proliferative specific functions (Figs. 1C, 2A and Supplement Table S1). The down-regulation of genes in the ECM and immune/inflammatory responses by isoproterenol also suggest a shift in preparation for cell proliferation and hypertrophy (Fig. 1C). Early down-regulation of ECM genes in rat parotid gland with isoproterenol treatment has

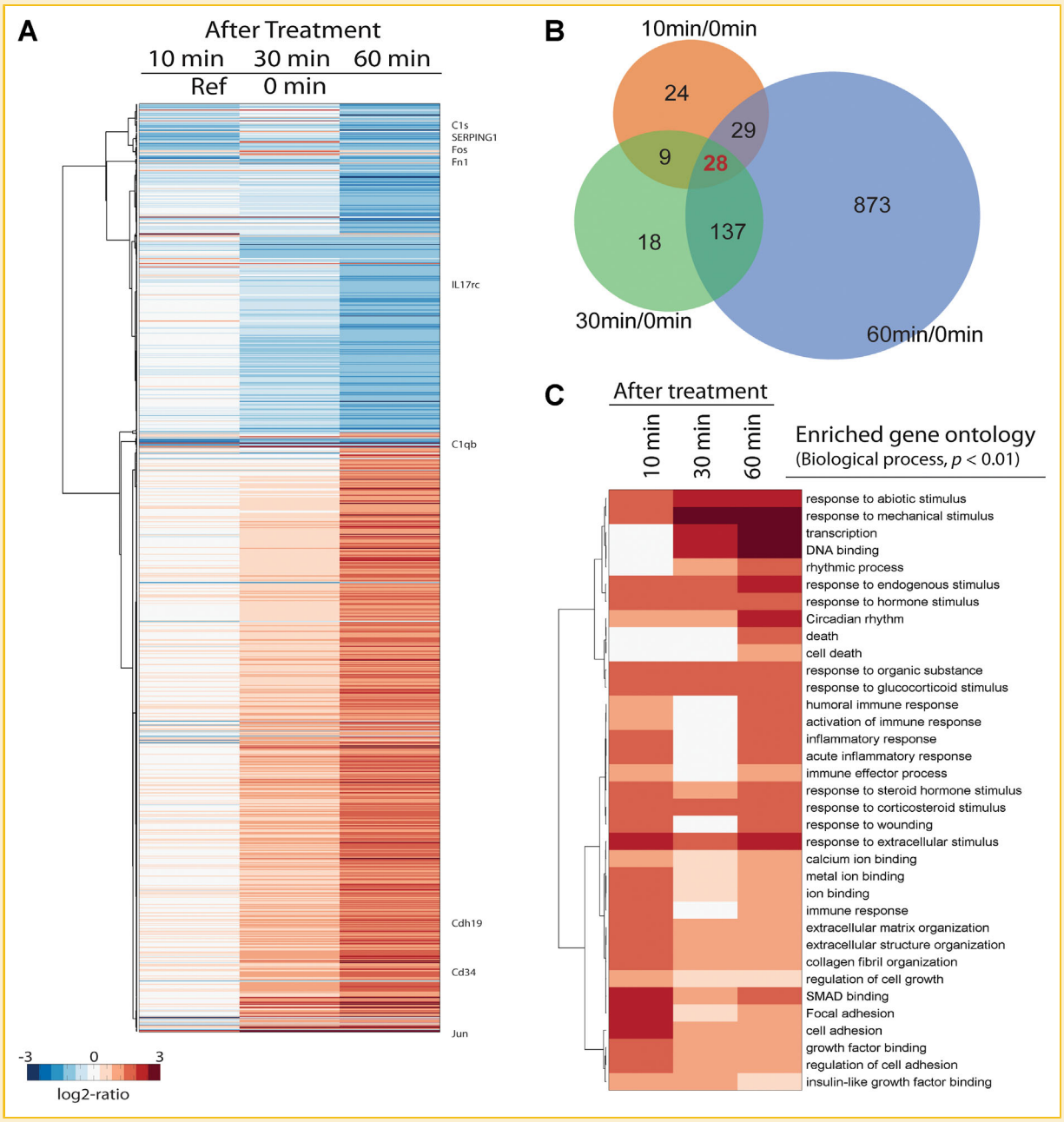

Fig. 1. Analysis of the genes that differently expressed between two groups after isoproterenol treatment for 10, 30, and 60 min. A: Hierarchical clustering of 1,117 genes' expression levels; B: Vann diagram of differential expressed genes in three comparisons against reference time point at 0: The probability of a DEG gene with log2-fold-changeis is $9.64 \times 10\text{-}6$ (see Statistic analysis) suggesting that the probability of observing 90 DEGs at 10 min purely due to random is near 0; and (C) Biological processes (Gene Ontology) enriched in differential expressed genes. Processes with enrichment *P*-value less than 0.01 were selected. White: not significant, dark-brown: highly significant.

previously been reported [Broverman et al., 1998]. Isoproterenol-mediated gland growth appears to be delicately regulated by proliferative and anti-proliferative genes. For example, up-regulation of the cell proliferative gene *jun b* [Yogev and Shaulian, 2010] and the down-regulation of the tumor suppressor genes *Igfbp3* [Werner and Katz, 2004], *Igfbp6* [Raykha et al., 2013], and *Igfbp7* [Liu et al., 2012] would favor cell proliferation. However, some anti-proliferative genes, for example, *Rasd1* [Vaidyanathan et al., 2004], *Wisp2* [Sabbah et al., 2011], and *Btg2* [Paruthiyil et al., 2011] were also up-regulated.

Ingenuity Pathway functional analysis highlighted the fact that many isoproterenol-regulated early genes are associated with cell growth/proliferation and cell death/survival functions (Supplement Table S1). These genes include *c-fos*, *Jun b*, *Btg2*, *Gadd45b*, *per2*, *Ptprb*, *Hsd11b1*, *Cyr61*, and *Mmp14*. We and others have shown that *c-fos* and *jun b* are early up-regulated transcription factors in

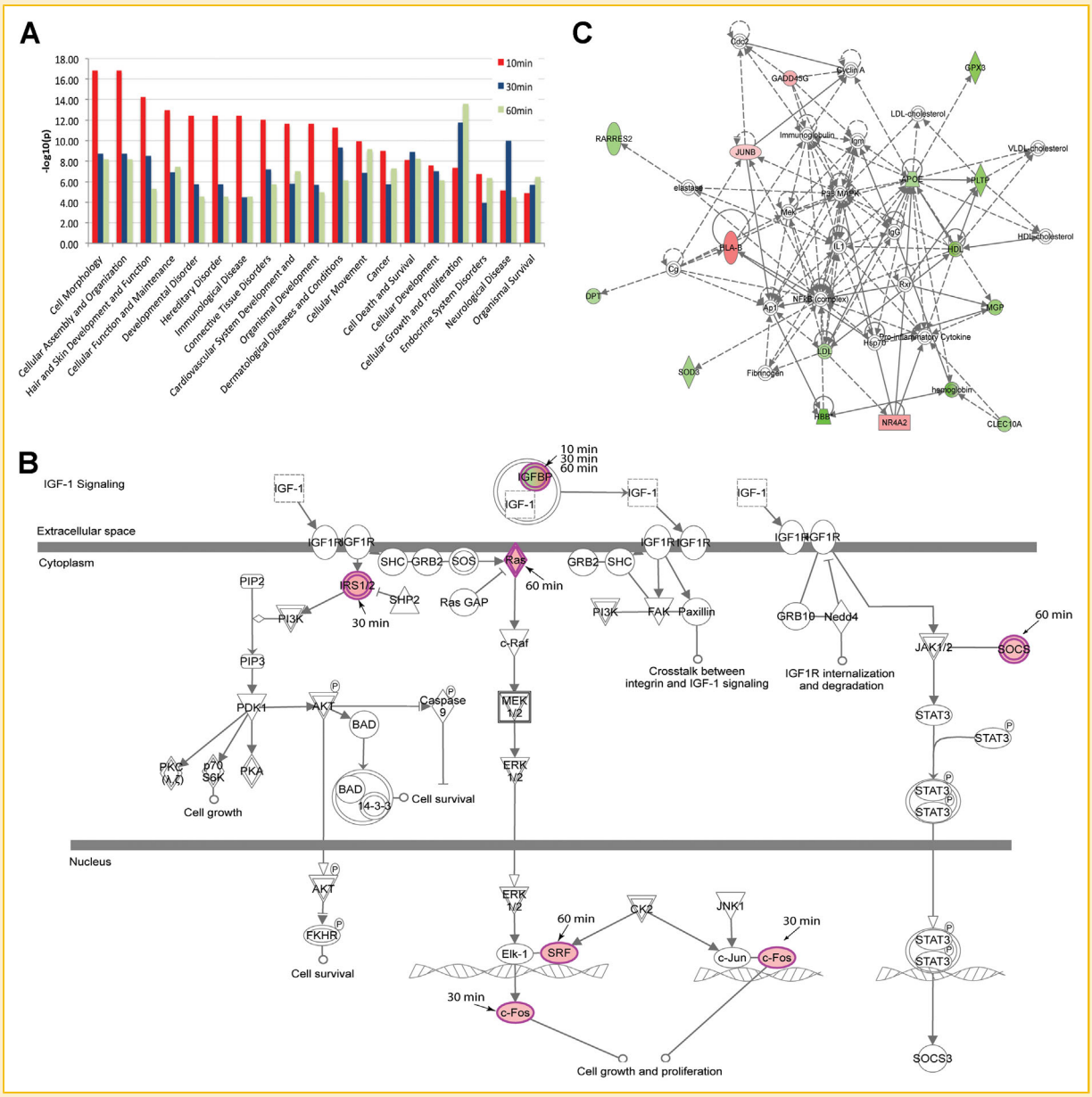

Fig. 2. Functional enrichment of differentially expressed genes in the salivary glands after isoproterenol treatment. A: Top 10 enriched relevant biological functions and diseases by IPA from three different time-points after isoproterenol treatment. Red, blue, and light green bars show the enrichment significance (−log10[p]) from 10 min, 30 min, and 60 min, respectively. The function "cell morphology" was most significant functions after isoproterenol treatment at 10 min (see Supplement Table S1). B: Insulin-like growth factor signaling pathways. Elements shaded by red color indicate significant changes after 10, 30, and/or 60 min (marked by small arrows). Note the Igfbp are down-regulated in all 3 time-points. C: The genes linked to p38MAPK that were differentially under-expressed (green) or overexpressed (red) 10 min after isoproterenol treatment. Note that although p38MAPK is at the center of the network, genes contained in the complex were not differentially expressed (open circle).

response to isoproterenol in salivary glands [Kousvelari et al., 1990; Yeh et al., 1991; Lee et al., 1992]. Other regulated genes have not been specifically evaluated in isoproterenol induced salivary gland enlargement. Among these genes, *Gadd45b* is an anti-apoptotic factor [Yamamoto and Negishi, 2008]; *Per2* is a tumor suppressor [Yamamoto and Negishi, 2008]; *Cyr61* promotes cell proliferation/survival/angiogenesis [Lau, 2011]; *Mmp14* is associated with tumorigenesis and metastasis [Egeblad and Werb, 2002]; and *Ptprb* is linked to inhibition of cell proliferation [Meng et al., 2000]. The down regulation of matrix metalloproteinases (MMPs) has been suggested to play a role in isoproterenol induced salivary gland hypertrophy and hyperplasia [Broverman et al., 1998]. The product of *Hsd11b1* may induce cell proliferation by inhibiting endogenous steroidogenesis [Yu et al., 2012]. The results of the current analysis demonstrate that within 60 min isoproterenol differentially affects genes involved in proliferation/tumorigenesis (upregulation of *c-fos*, *jun b*, *Btg2*, *Gadd45b*, *Cyr61*, and *Hsd11b1* and down-regulation of *Per2*, *Ptprb*, and *Mmp14*). The balance of expression

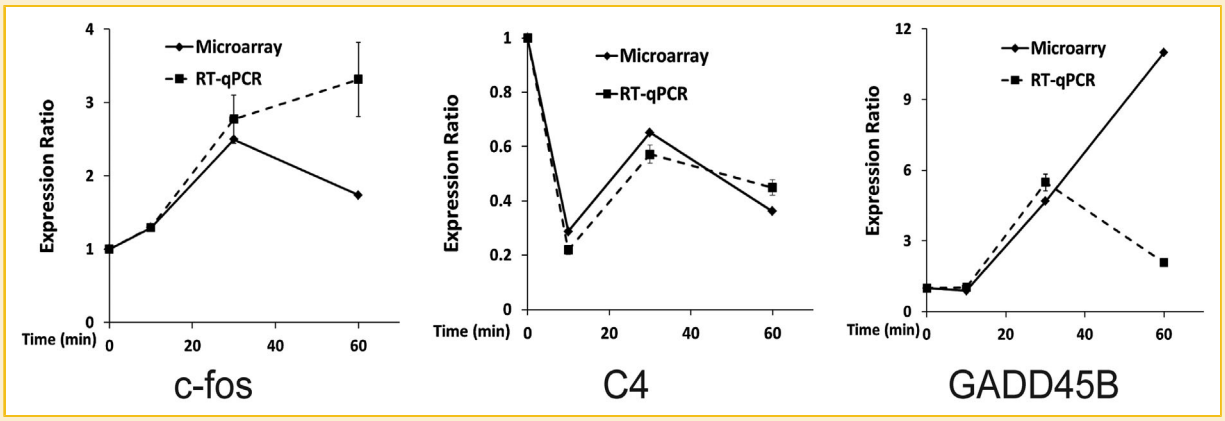

Fig. 3. Gene expression in microarray was confirmed by quantitative real-time PCR. Expression of the genes c-fos, Gadd45b, C4 was significantly different from the expression in the salivary glands of without isoproterenol treatment. Error bar = SE of RT-qPCR results. The oligonucleotide primer sequences used: c-fos pair forward 5′-GGGAGTGGTGAAGACCA-TGT-3′; reverse 5′-CGCTTGGAGCGTATCTGTC-3′; C4 pair forward 5′-CAGGAGGTGAAAGGCTCAGT-3′; reverse 5′- CAAAGAGGCCACAACTCCTC-3′; GADD45B forward 5′- CGGCCAAACTGATGAATGT-3; reverse 5′-GGGTGAAGTGAATTTGCAGAG-3′; GADPH forward 5′-TA-TGACTCTACCCACGGCAAGT-3; reverse 5′-ATACTCAGCACCAGCATCACC-3′. Gene expression was then normalized using the housekeeping gene GAPDH.

between these genes may play a critical role in limiting the extent of salivary gland enlargement in response to isoproterenol.

Ingenuity Pathway Analysis identified three different pathways, each at a different time post-injection, affected by isoproterenol. At 10 min, the complement system was the top pathway. Complement is an innate immune defense pathway leading to cell lysis and death or the activation of calcium signaling to induce cell proliferation [Tegla et al., 2011; Qiu et al., 2012]. Therefore, down-regulation of complement (*C1q*, *C1r*, *C1s*, and *C4*) as an early event in isoproterenol-induced salivary gland enlargement warrants further investigation. The significance of the prothrombin activation pathway at 30 min after isoproterenol injection in salivary gland enlargement is less clear at this moment. At 60 min, the top pathway was the LPS/IL-1 mediated inhibition of RXR function, which can regulate genes associated with organ development/differentiation as well as tumorigenesis [Bushue and Wan, 2010], which may play a role in isoproterenol-induced gland enlargement. Canonical pathway analysis also showed an enriched IGF-1 pathway. Salivary gland produces IGF-1 and IGF-1 receptor. After isoproterenol treatment, IGF-1 may enhance salivary cell proliferation/survival through the IGF-1 pathway with down-regulation of IGFBPs 3, 6, and 7 which are known to be anti-proliferative [Werner and Katz, 2004].

Our prior studies have shown an activation of the mitogen activated protein kinase (MAPK) pathways, including ERK1/2 and p38MAPK, in rat parotid gland [Yeh et al., 2012]. In the current study, $p38^{MAPK}$ is one of the top three networks identified by IPA in isoproterenol-treated rat salivary glands. Activation of $p38^{MAPK}$ has been associated with cell proliferation, differentiation and cell survival in response to external stimuli [Schwindinger and Robishaw, 2001; Thornton and Rincon, 2009]. $p38^{MAPK}$ inhibitors have been shown to protect acinar cells from de-differentiation in primary cultures of rat parotid gland [Fujita-Yoshigaki et al., 2008]. Among the genes related to the network, *Jun b* and *Gadd45B* were significantly elevated. Jun B is significantly elevated in salivary gland cells after isoproterenol treatment [Yeh et al., 1991]. Gadd45b may inhibit the $p38^{MAPK}$ upstream activator MTK1/MEKK4 kinase or Mkk7 to mediate an anti-apoptotic and/or proliferative function [Takekawa and Saito, 1998; Yamamoto and Negishi, 2008].

TABLE I. Number of Differentially Expressed Genes (>2 fold changes) After Isoproterenol Treatment

Times	Total genes	Up-regulated genes	Down-regulated genes
10 min	90	17	73
30 min	192	120	72
60 min	1067	696	371

Changes in gene expression in mouse parotid glands, after systemic administration of isoproterenol, have been shown to be complex and time-dependent (30 min to 48 h) [Ten Hagen et al., 2002]. Here, we have confirmed the presence and identity of very early gene changes in rat parotid gland in response to isoproterenol stimulation using complementary bioinformatic analyses. We have tentatively identified a limited number of DEGs, produced in response to isoproterenol treatment, which represent an initial step in elucidating the mechanism of action of isoproterenol in salivary gland enlargement. Patients treated with isoproterenol or β2-adrenoceptor agonists have experienced salivary gland enlargement/swelling and changes in salivary gland function [Borsanyi and Blanchard, 1962; Ryberg et al., 1991; Minakami et al., 1992], suggesting that chronic isoproterenol induced salivary gland enlargement in rodents is an appropriate correlate for studying changes in human salivary glands. An understanding of the early changes in gene expression, which represent a delicate balance between gene up- and down regulation (e.g., *c-fos*, *Jun b*, *Cyr61*, *Btg2*, *Gadd45b*, *Per2*, *Ptprb*, *Hsd11b1*, and *Mmp14*) and various activated pathways (e.g., IGF-1 and p38MAPK) involving hyperplasia, proliferation, and apoptosis, may provide an explanation for the self-limited gland enlargement found with isoproterenol. Further studies, using experimental approaches similar to those described in this report, will help determine how salivary

glands may be regenerated and identify pathways activated in salivary gland tumorigenesis.

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REFERENCES

Barka T. 1990. B-Adrenergic effects on salivary glands: Growth and gene regulation. Acta Histochem Cytochem 23:245–255.

Borsanyi SJ, Blanchard CL. 1962. Asymptomatic parotid swelling and isoproterenol. Laryngoscope 72:1777–1783.

Broverman RL, Nguyen KH, da SA Brinkley, MacAuley SP, Zeng T, Yamamoto H, Tarnuzzer RW, Schultz GS, Kerr M, Humphreys-Beher MG. 1998. Changes in the expression of extracellular matrix (ECM) and matrix metalloproteinases (MMP) of proliferating rat parotid acinar cells. J Dent Res 77:1504–1514.

Buchner A, Sreebny LM. 1972. Enlargement of salivary glands: Review of the literature. Oral Surg 24:209–222.

Bushue N, Wan YJ. 2010. Retinoid pathway and cancer therapeutics. Adv Drug Deliv Rev 62:1285–1298.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174.

Fujita-Yoshigaki J, Matsuki-Fukushima M, Sugiya H. 2008. Inhibition of Src and p38 MAP kinases suppresses the change of claudin expression induced on dedifferentiation of primary cultured parotid acinar cells. Am J Physiol Cell Physiol 294:C774–C785.

Grotendorst GR, Lau LF, Perbal B. 2001. CCN proteins are distinct from, and should not be considered members of, the insulin-like growth factor-binding protein superfamily. J Clin Endocrinol Metab 86:944–945.

Huang dW, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57.

Kousvelari E, Yeh CK, Mertz PM, Chinchetru M. 1990. Regulation of proto-oncogenes and salivary gland cell proliferation. Adv Dent Res 4:61–68.

Lau LF. 2011. CCN1/CYR61: The very model of a modern matricellular protein. Cell Mol Life Sci 68:3149–3163.

Lee SW, Purushotham KR, Littlewood T, Evan G, Zelles T, Blazsek J, Nakagawa Y, Humphreys-Beher MG. 1992. Down-regulation of cellular proto-oncogenes during inhibition of rat parotid acinar cell proliferation. Biochim Biophys Acta 1135:115–122.

Liu ZK, Liu HY, Fang WN, Yang Y, Wang HM, Peng JP. 2012. Insulin-like growth factor binding protein 7 modulates estrogen-induced trophoblast proliferation and invasion in HTR-8 and JEG-3 cells. Cell Biochem Biophys 63:73–84.

Matsuura S, Suzuki K. 1997. Immunohistochemical analysis of DNA synthesis during chronic stimulation with isoproterenol in mouse submandibular gland. J Histochem Cytochem 45:1137–1145.

Meng K, Rodriguez-Pena A, Dimitrov T, Chen W, Yamin M, Noda M, Deuel TF. 2000. Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. Proc Natl Acad Sci USA 97:2603–2608.

Minakami H, Takahashi T, Izumi A, Itoi H, Tamada T. 1992. Enlargement of the salivary gland after ritodrine treatment in pregnant women. Br Med J 304:1668.

Ng WF, Bowman SJ. 2010. Primary Sjogren's syndrome: Too dry and too tired. Rheumatology (Oxford) 49:844–853.

Paruthiyil S, Cvoro A, Tagliaferri M, Cohen I, Shtivelman E, Leitman DC. 2011. Estrogen receptor beta causes a G2 cell cycle arrest by inhibiting CDK1 activity through the regulation of cyclin B1, GADD45A, and BTG2. Breast Cancer Res Treat 129:777–784.

Qiu W, Zhang Y, Liu X, Zhou J, Li Y, Zhou Y, Shan K, Xia M, Che N, Feng X, Zhao D, Wang Y. 2012. Sublytic C5b-9 complexes induce proliferative changes of glomerular mesangial cells in rat Thy-1 nephritis through TRAF6-mediated PI3K-dependent Akt1 activation. J Pathol 226:619–632.

Raykha C, Crawford J, Gan BS, Fu P, Bach LA, O'Gorman DB. 2013. IGF-II and IGFBP-6 regulate cellular contractility and proliferation in Dupuytren's disease. Biochim Biophys Acta 1832:1511–1519.

Ryberg M, Moller C, Ericson T. 1991. Saliva composition and caries development in asthmatic patients treated with b_2-adrenoceptor agonists: A 4-year follow-up study. Scand J Dent Res 99:212–218.

Sabbah M, Prunier C, Ferrand N, Megalophonos V, Lambein K, De WO Nazaret, Lachuer J, Dumont S, Redeuilh G. 2011. CCN5, a novel transcriptional repressor of the transforming growth factor beta signaling pathway. Mol Cell Biol 31:1459–1469.

Schwindinger WF, Robishaw JD. 2001. Heterotrimeric G-protein βγ-dimers in growth and differentiation. Oncogene 20:1653–1660.

Selye H, Veilleux R, Cantin M. 1961. Excessive stimulation of salivary gland growth by isoproterenol. Science 133:44–45.

Takekawa M, Saito H. 1998. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 95:521–530.

Tegla CA, Cudrici C, Patel S, Trippe R, III., Rus V, Niculescu F, Rus H. 2011. Membrane attack by complement: The assembly and biology of terminal complement complexes. Immunol Res 51:45–60.

Ten Hagen, Balys KG, Tabak MM, Melvin LA. 2002. Analysis of isoproterenol-induced changes in parotid gland gene expression. Physiol Genomics 8:107–114.

Thornton TM, Rincon M. 2009. Non-classical p38 map kinase functions: Cell cycle checkpoints and survival. Int J Biol Sci 5:44–51.

Vaidyanathan G, Cismowski MJ, Wang G, Vincent TS, Brown KD, Lanier SM. 2004. The Ras-related protein AGS1/RASD1 suppresses cell growth. Oncogene 23:5858–5863.

Werner H, Katz J. 2004. The emerging role of the insulin-like growth factors in oral biology. J Dent Res 83:832–836.

Yamamoto Y, Negishi M. 2008. The antiapoptotic factor growth arrest and DNA-damage-inducible 45 beta regulates the nuclear receptor constitutive active/androstane receptor-mediated transcription. Drug Metab Dispos 36:1189–1193.

Yeh CK, Chandrasekar B, Lin AL, Dang H, Kamat A, Zhu B, Katz MS. 2012. Cellular signals underlying beta-adrenergic receptor mediated salivary gland enlargement. Differentiation 83:68–76.

Yeh CK, Chinchetru MA, Kousvelari E. 1991. Characteristics of c-fos and jun B gene expression in A5 cells after beta-adrenoreceptor stimulation and during the cell cycle. Biochim Biophys Acta 1090:173–180.

Yogev O, Shaulian E. 2010. Jun proteins inhibit autophagy and induce cell death. Autophagy 6:566–567.

Yu Y, Wei N, Stanford C, Schmidt T, Hong L. 2012. In vitro effects of RU486 on proliferation and differentiation capabilities of human bone marrow mesenchymal stromal cells. Steroids 77:132–137.

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