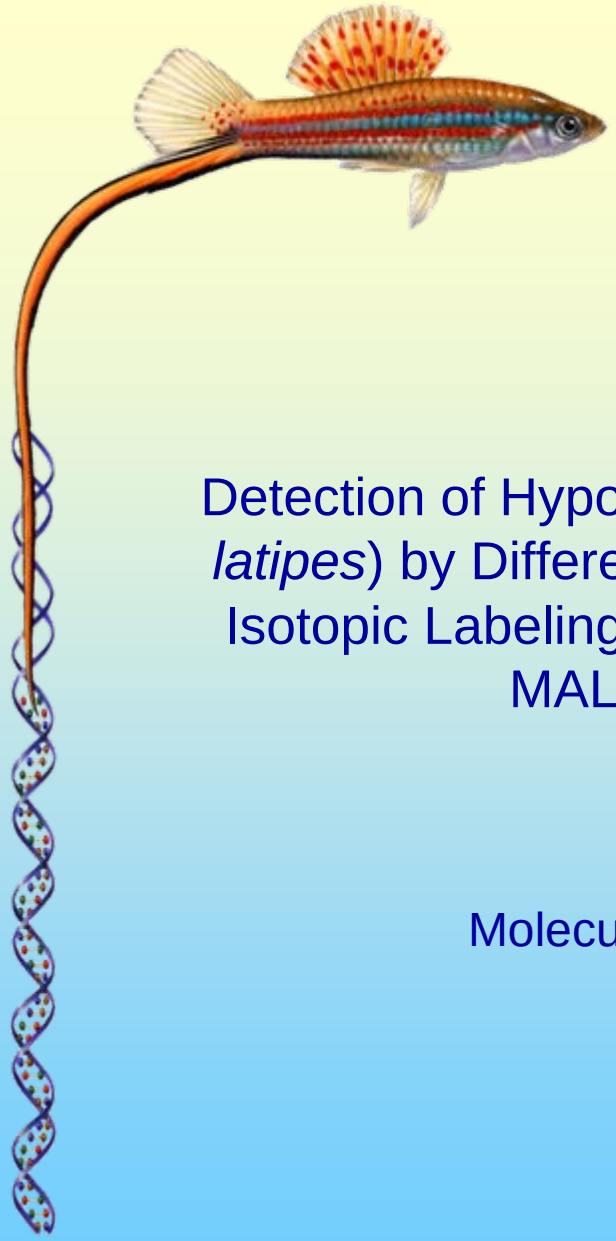

Detection of Hypoxia-Related Proteins in Medaka (*Oryzias latipes*) by Difference Gel Electrophoresis and Differential Isotopic Labeling Combined with Multi-dimensional μLC-MALDI-TOF Mass Spectrometry

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Overview

- Difference Gel Electrophesis (DIGE)
  - What is DIGE?
  - Identifying candidate hypoxia biomarkers
    - Brain
    - Fin
- geLC-MALDI-PSD
  - What is geLC?
  - Identifying candidate hypoxia biomarkers
    - Cultured cells
    - Liver
- Conclusions and future plans

Medaka hypoxia exposure conditions:

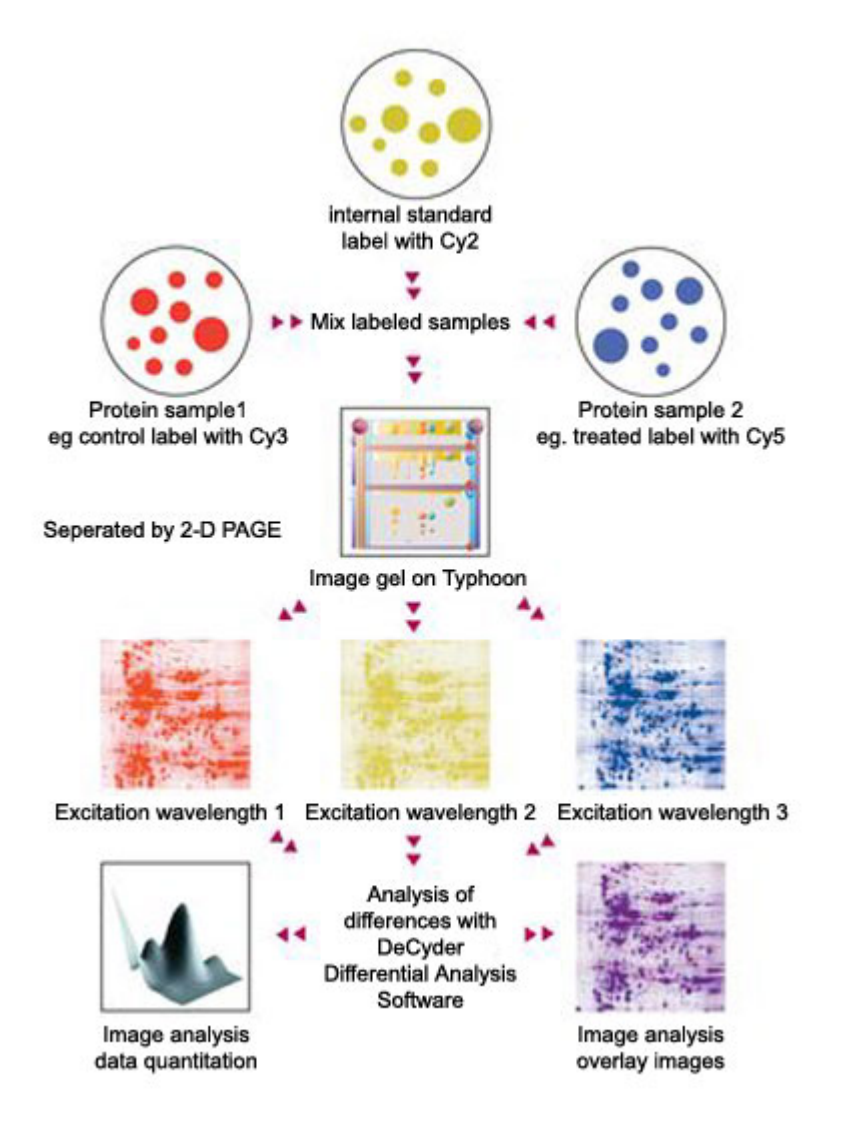
- Four 20-gallon aquaria used - two for control and two for hypoxia.
- Each tank contained 18 adult male medaka.
- Hypoxia exposure duration of one day at 2.85 dissolved oxygen (DO), one day at 2.38, two days at 1.90, followed by five days at .95 DO.
- Extract RNA and protein from same tissues from both control and hypoxic for microarray, RT-PCR, and proteomic analyses.

Difference Gel Electrophoresis

Detection of hypoxia-related biomarker proteins in medaka brain tissue via two-dimensional difference gel electrophoresis (2D-DIGE):

- Differentially label control and treated fish brain extracts with Cy3 and Cy5, respectively.
- Include pooled internal standard labeled with Cy2 to minimize gel-to-gel variation.
- Combine labeled extracts and separate by two-dimensional gel electrophoresis (2D-PAGE).
- Normalize gel spot intensities and analyze for proteins showing statistically significant up- and down-regulation.

DIGE Overview

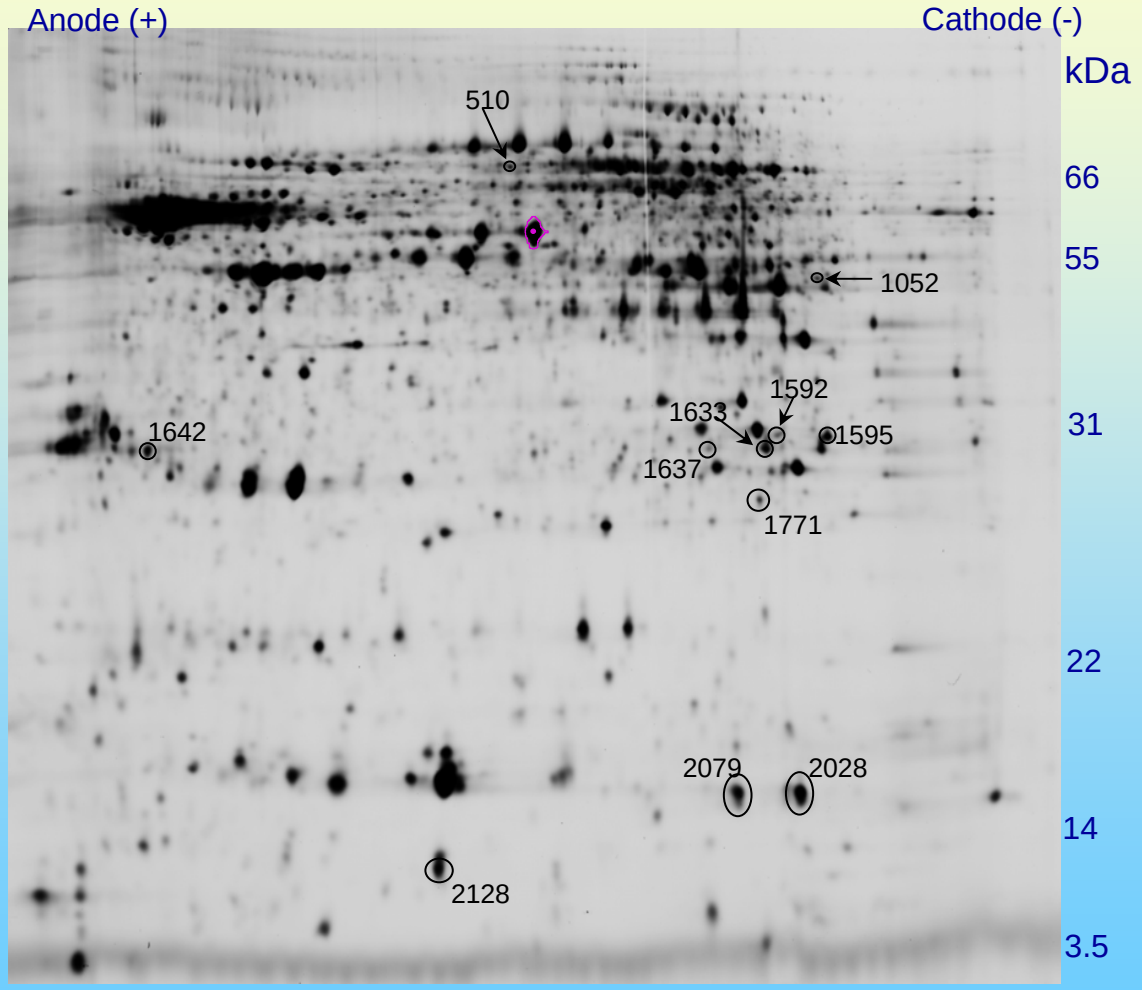


2D-DIGE gel data analysis

- Spot volumes across replicate gels and across “dye flips” were normalized to the Cy2 internal standard images.
- Spot volume ratios between hypoxic and control images were calculated.
- Gel spots exhibiting p =< 0.05 and 80% Power were selected as proteins of interest (POIs).

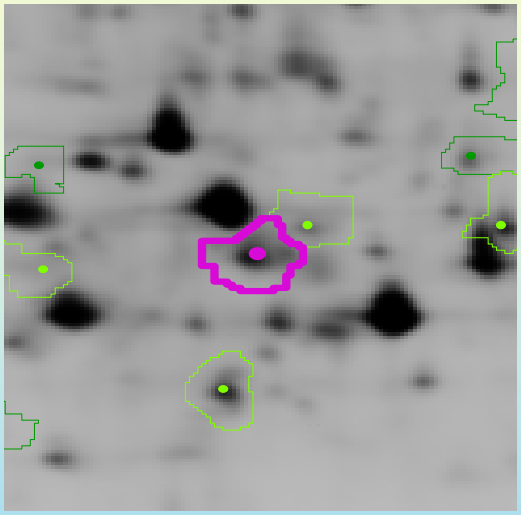
www.kyoiku-shuppan.co.jp/.../title.html

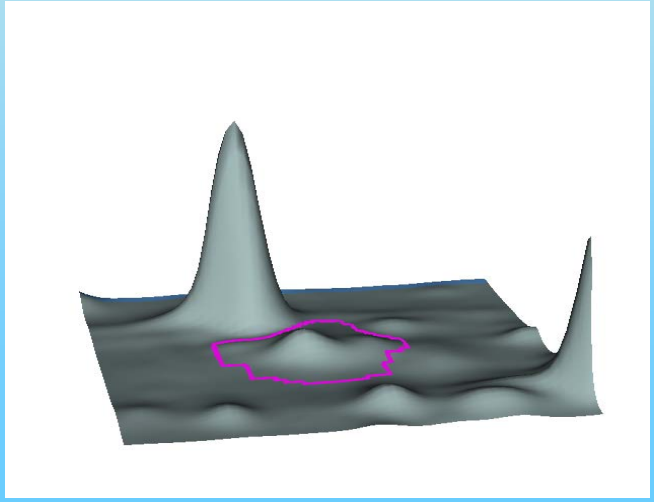
Brain 2D-DIGE gel spots up-regulated in response to hypoxia



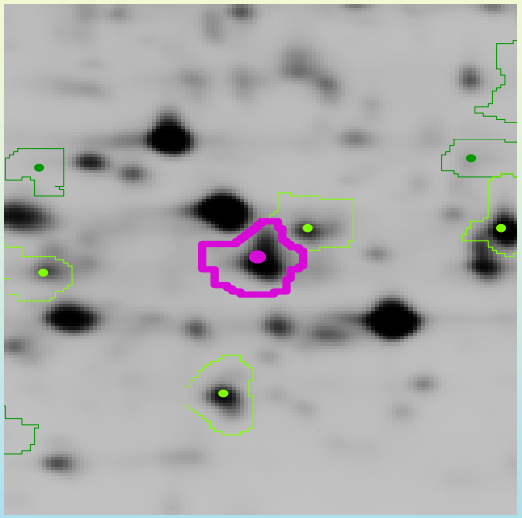
3-D Representation of Brain Protein Abundance Difference Upon Hypoxia Exposure

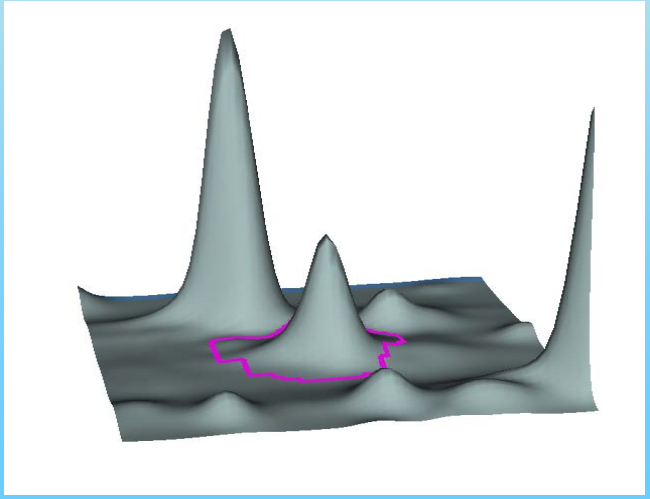
Normoxic



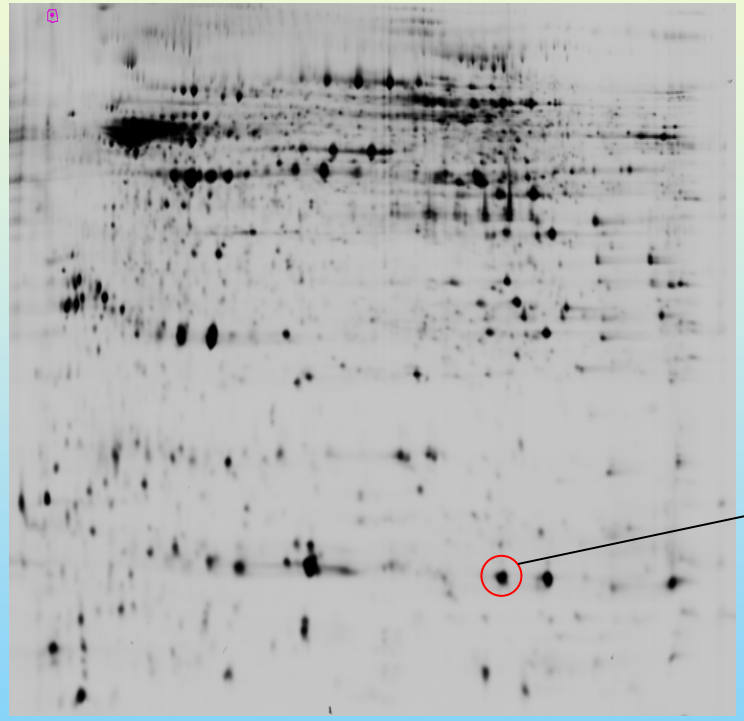


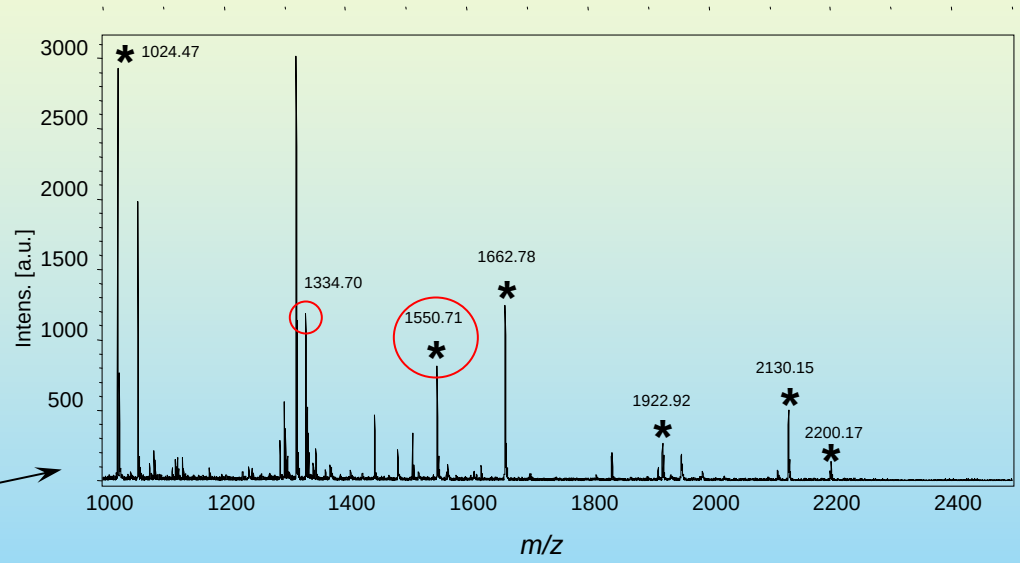
Hypoxic



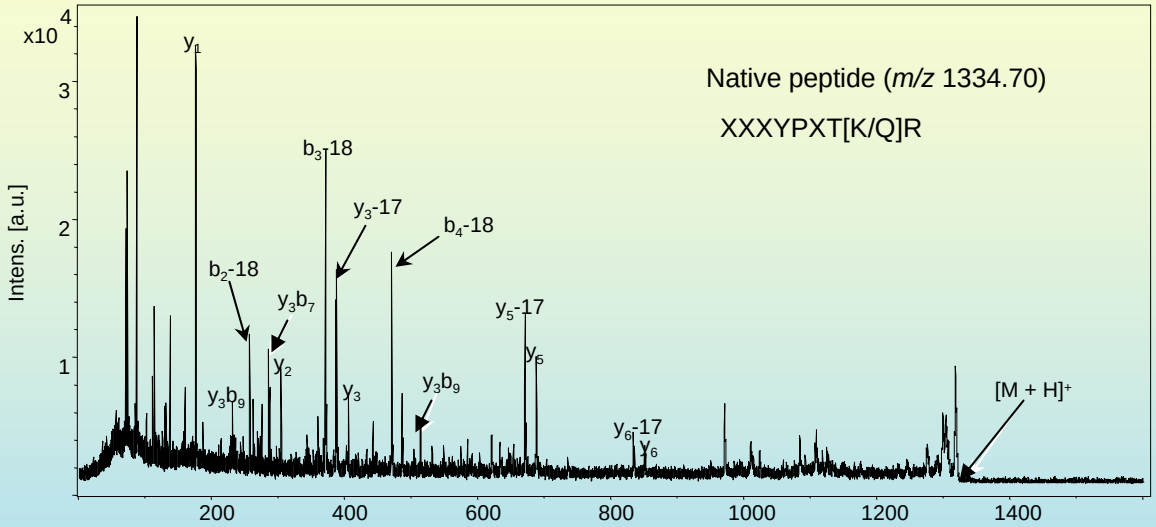


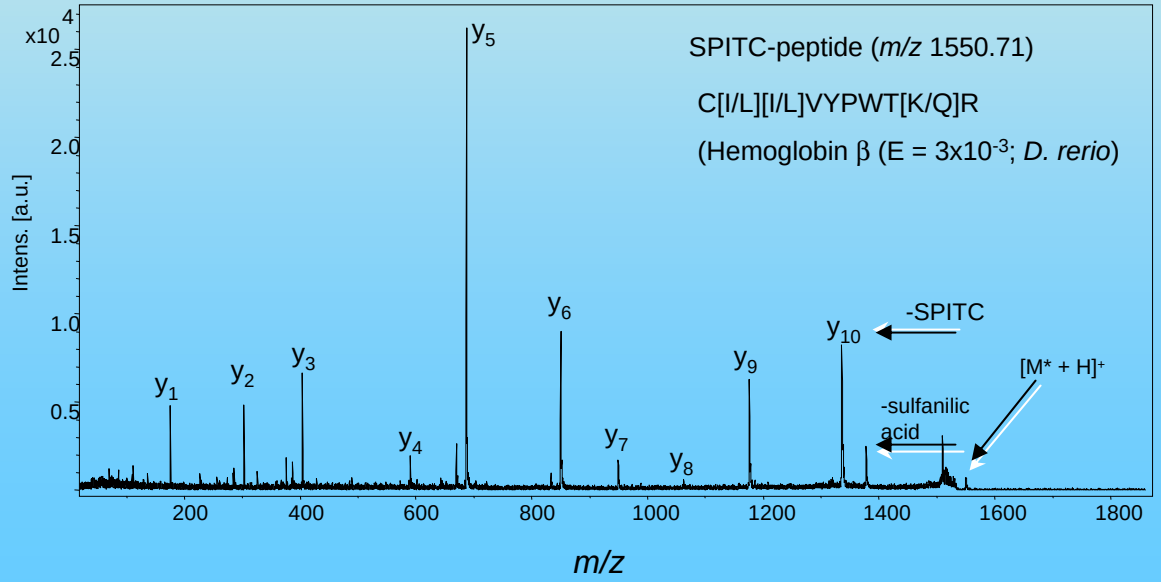
Example of Parent Spectra of Peptide Obtained From Protein # 2079



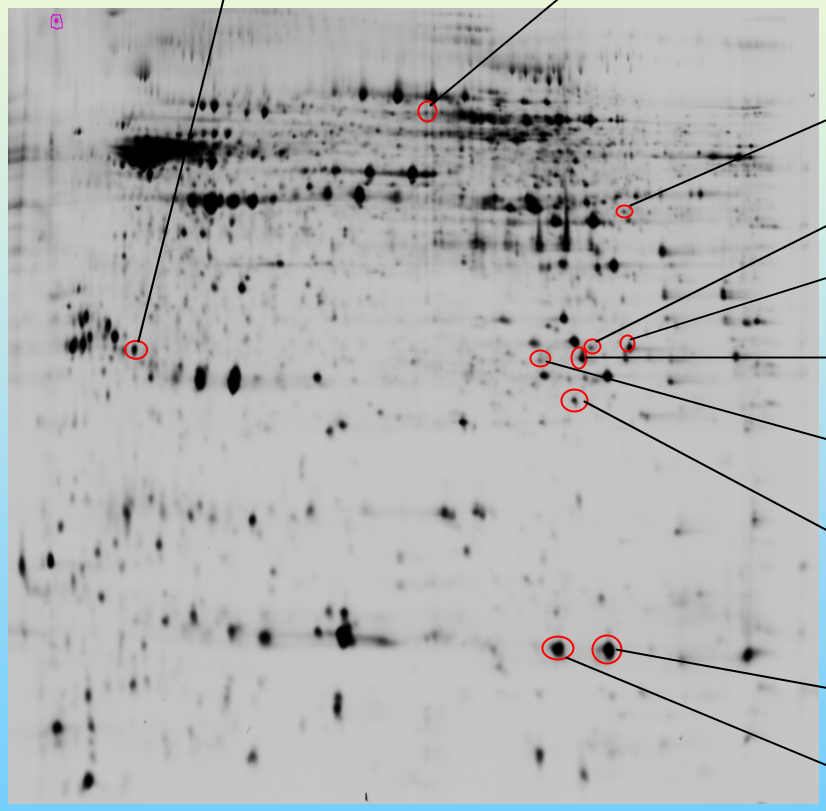


Postsource Decay of Peptides with and without SPITC Dervitization





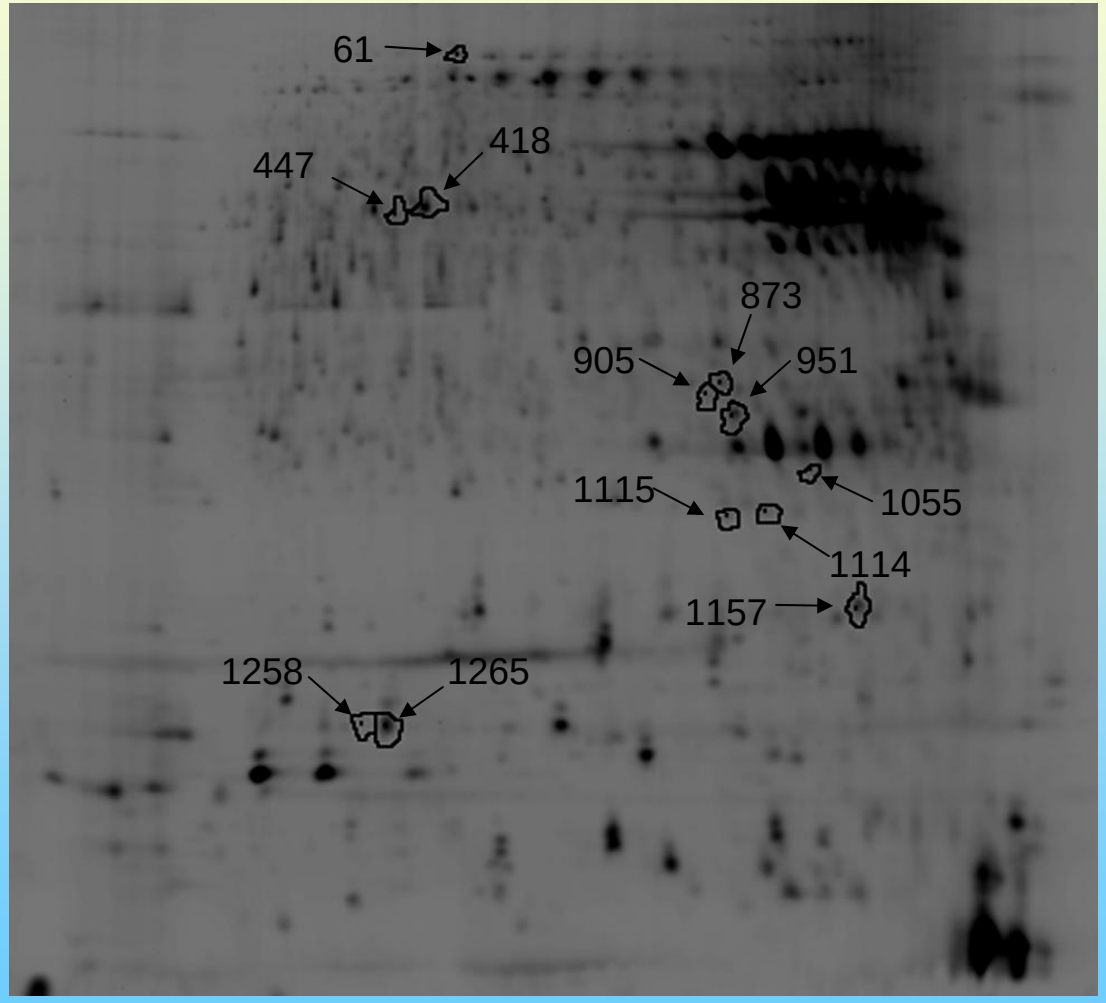
DIGE Medaka Brain Hypoxia Proteins of Interest with Tentative Identifications



DIGE-MALDI-TOF-PSD of Medaka brain tissue: 1.19-1.43 % DO; 192 hours

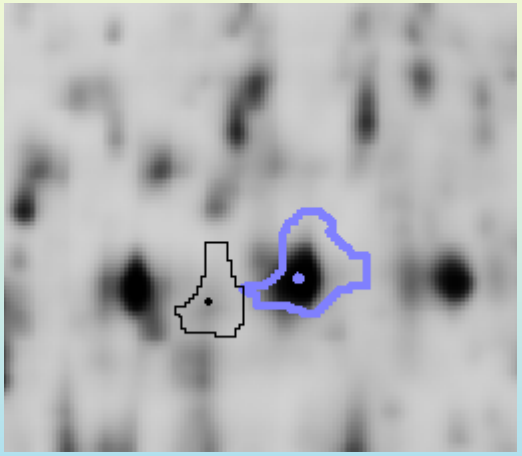
Protein	Hyp/Cntl	Implicated Function (based upon hypoxia literature)
hemoglobin	3.22	RBC production known to be induced under hypoxia through HIF-1 regulation of EPO and VEGF1.
carbonic anhydrase II (CA2)	2.09	CA2 upregulation under hypoxia resulting from increased RBC through HIF-mediated EPO and VEGF1 induction.
calbindin/calretinin	1.53	suspected to provide neuroprotective effects against hypoxia; function as an intracellular buffer to control Ca^{2+} toxicity under hypoxic conditions.
aldolase	1.51	A gene promoter contains HIF-1 binding site, likely induced by HIF-1. Aldolase induction shift from aerobic to anaerobic metabolism.
glutathione-S-transferase (GST)	1.50	GST known to be hypoxia/anoxia-regulated in a range of hypoxia-tolerant vertebrates. Likely protects cells from oxidative damage upon reperfusion.

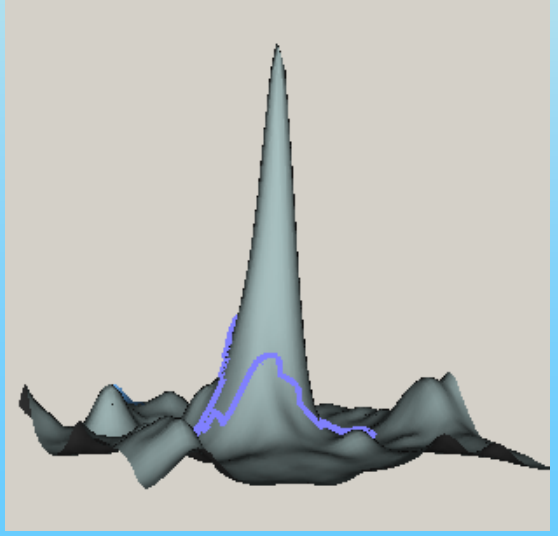
DIGE Medaka Fin Hypoxia Proteins of Interest



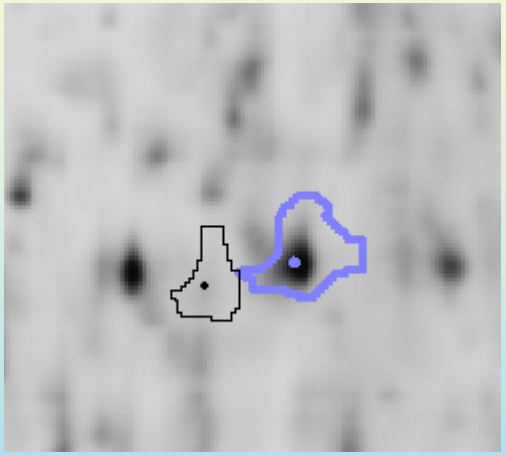
3-D Representation of Fin Protein Abundance Difference Upon Hypoxia Exposure

Hypoxic





Normoxic



DIGE Conclusions

- Brain proteins differentially expressed upon hypoxia exposure
  - 10 proteins of interest
  - Tentative protein identification
    - Succinate dehyrogenase, aldolase, carbonic anhydrase, calbindin, glutathione-S-transferase, hemoglobin

- Fin proteins differentially expressed upon hypoxia exposure
  - 12 proteins of interest
  - Protein identification through peptide sequencing by MALDI-TOF MS is in progress

- Future plans
  - Continue DIGE analysis on other tissues including gill and liver
  - Confirm biomarker identifications through western blot and/or real-time PCR

geLC MALDI-MS

- Pre-fractionate proteins.
- Differentially label peptides at C-termini with $H_2{}^{16}O$ (cntl) or $H_2{}^{18}O$ (hyp).
- Sulfonate peptides at N-termini with 4-sulfophenyl isothiocyanate (SPITC).
- Fractionate peptides by reversed-phase μLC.

geLC-MALDI-PSD workflow

Extract protein from medaka cells or tissues

↓

Prefractionate control/treated proteins by SDS-PAGE

↓

Excise 6 gel bands spanning the PAGE molecular weight range.

↓

Perform in-gel trypsin digestion of gel-entrained proteins

↓

Extract tryptic peptides from gel plugs

↓

Differentially label peptides with $H_2{}^{16}O$ (control) and $H_2{}^{18}O$ (hypoxic)

↓

Combine peptides and derivatize with SPITC

↓

Separate peptides by reversed-phase nanoflow μLC

↓

Collect fractions directly onto MALDI targets

↓

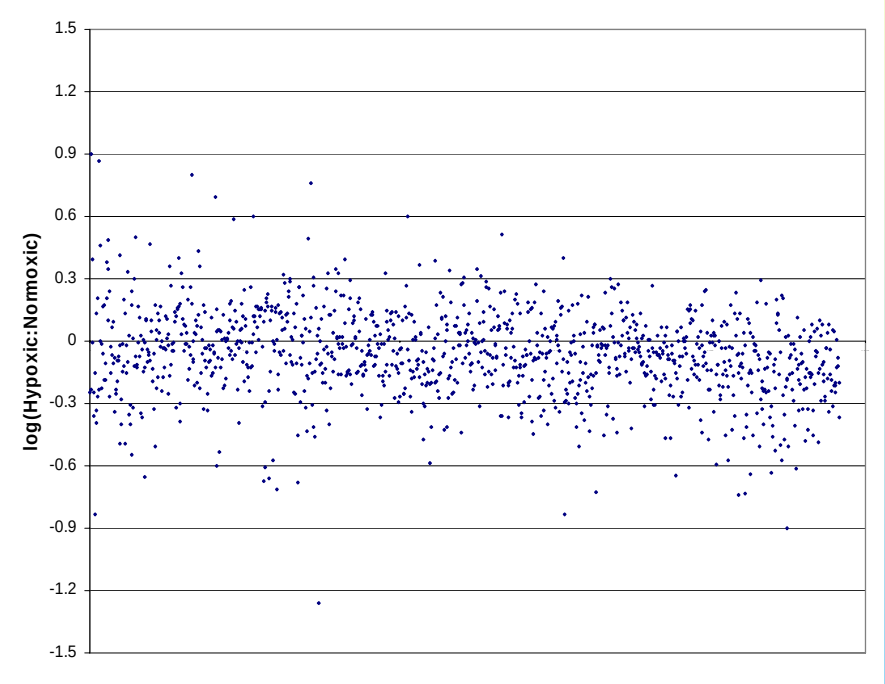
Collect parent ion spectra and identify peptide ion pairs-of-interest

↓

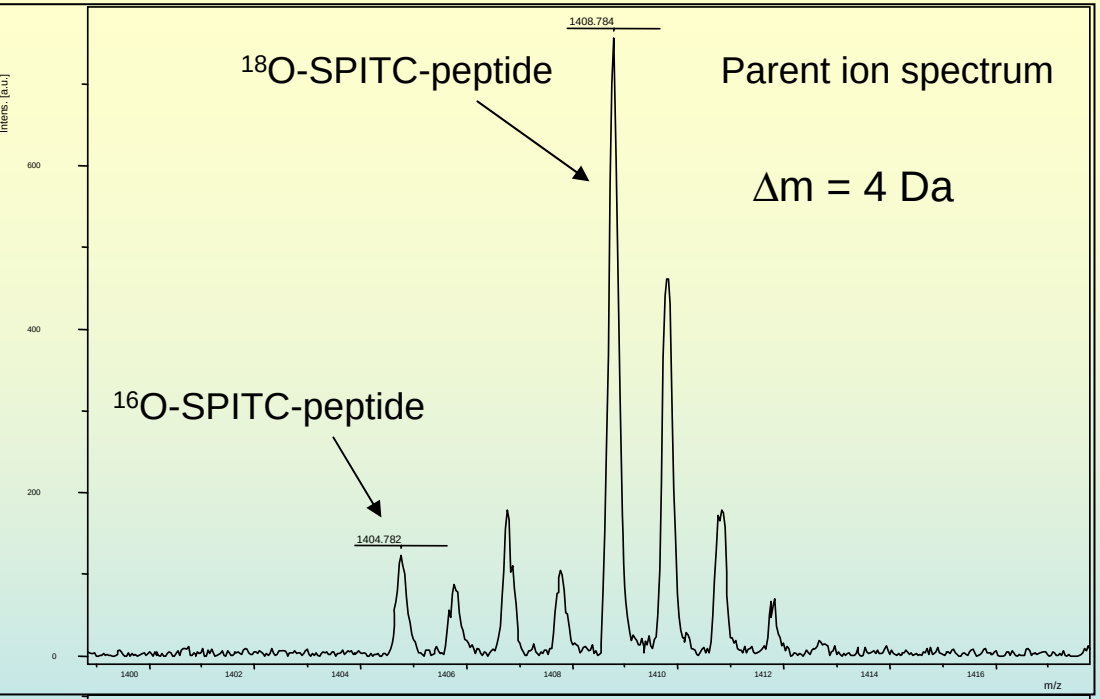
Normalize data and sequence peptides-of-interest by MALDI-PSD

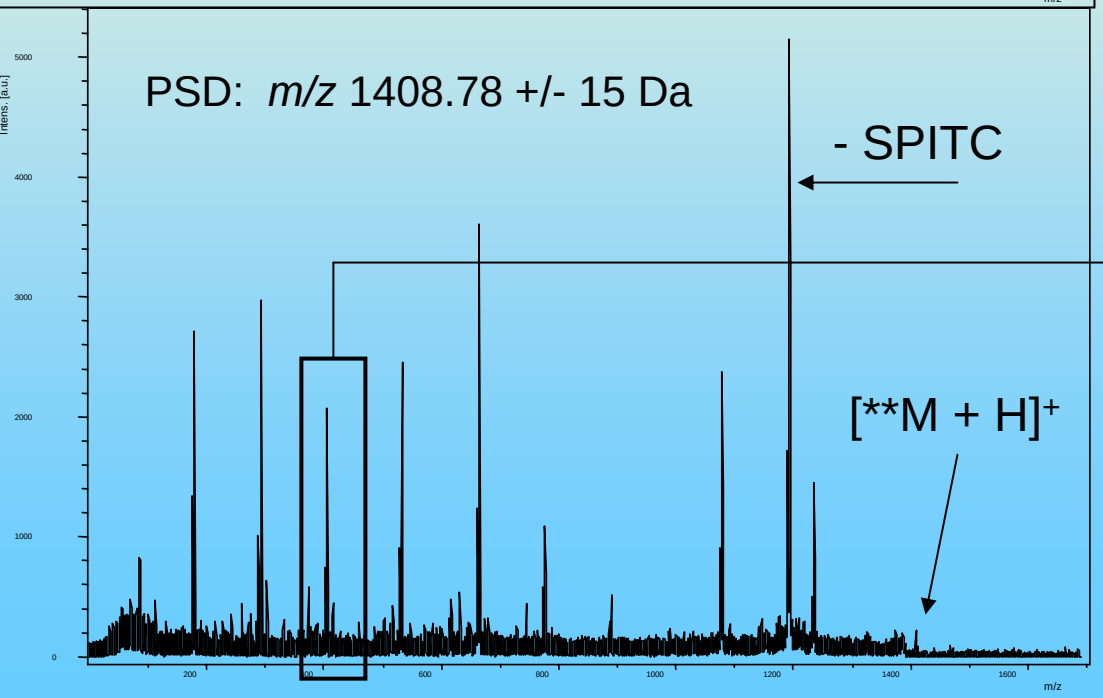
geLC-MALDI-MS of medaka cultured cells exposed to .95 dissolved oxygen for 120 hrs

- 6 SDS-PAGE MW fractions used.
- Each PAGE fraction peptide population was separated on a C18 capillary column using a 180 min 0-70% ACN gradient (total of 840 minutes µLC fractionation time).
- Collected a total of 384 µLC fractions onto MALDI target using Probot fraction collector.
- Estimated ~4000 resolved peptides.
- 1210 unique and well-resolved $^{16}O/^{18}O$ peptide pairs were detected by inspection of relative isotopic abundances.
- Calculated normalized abundance ratios for each $^{16}O/^{18}O$ pair.
- Collected PSD spectra for any peptide ion pair showing >= 2.00 hypoxic:normoxic ratio.
- Sequences were deduced only from high-quality PSD spectra.

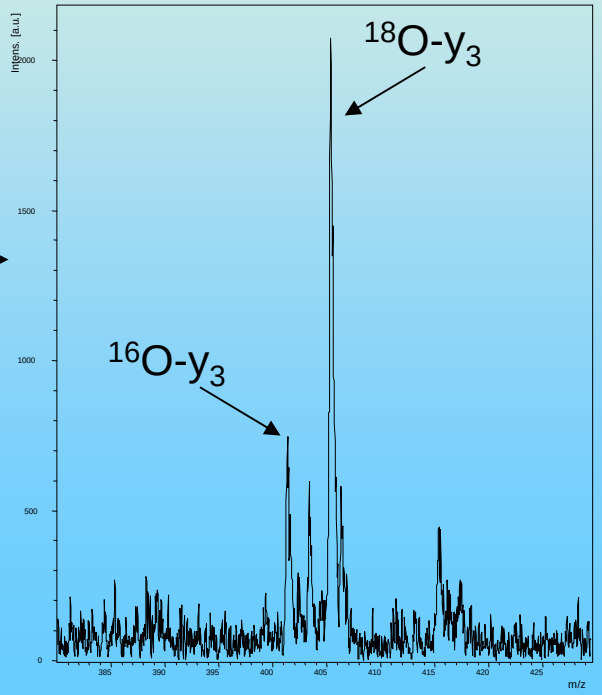


Example spectra: up-regulated protein

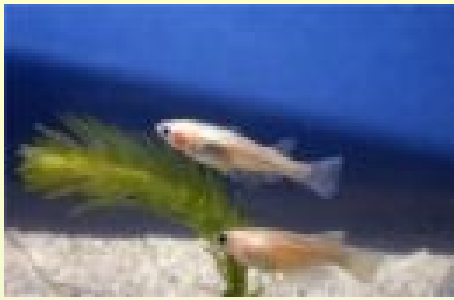




y_3 fragment ion



Results: geLC-MALDI-PSD



*park.itc.u-tokyo.ac.jp/K-**medaka**/AS-CV-J*

- $^{16}O/^{18}O$ labeling plus sulfonation provides a powerful method for quantitation and sequence analysis.
- PSD provides additional confirmation of a differentially labeled peptide pair; reduces chance inclusion of unrelated peptides differing by 4 Da in mass.
- Co-eluting peptides can still be quantified and sequenced by examining relative $^{16}O/^{18}O$ ratios of respective fragment ion peaks in PSD spectra.
- Thus, PSD provides an additional 3rd dimension of separation.
- Unambiguous and unique peptide sequences provide high information content even in absence of accurate mass comparisons with translated genome database.

Medaka cultured cells: peptide sequences and BLAST results

m/z	Hyp/Norm	Sequence	Best BLAST match
1421.81	**11.6**	**GLVHNNMRGGR**	**predicted similar to mKIAA0821 protein (E = 0.43; D. rerio)**
1387.74	**7.36**	**QVHPDTGLFR**	**unknown**
1671.81	**5.77**	**TASPQQAQEV**	**triosephosphate isomerase B (E = 0.013; D. rerio)**
1330.97	**4.90**	**GDENNGLTHR**	**predicted similar cadherin EGE LAG seven-pass G-type receptor 3 (E = 1.0; D. rerio)**
1130.61	**4.01**	**SVCF(LE,YL)R**	**unknown**
1800.00	**3.96**	**NTQLDFNFNALLR**	**forkhead protein FKHR (E = 1.0; D. rerio)**
1130.67	**3.86**	**LLMEFLR**	**sequestosome 1 (E = 7.6; D. rerio)**
1396.60	**3.25**	**FALSQLLTQR**	**unknown**
1446.73	**2.92**	**GNHVAQVYALR**	**novel protein similar to vertebrate ribosomal protein S16 (E = 0.18; D. rerio)**
1434.81	2.52	VFVGTTLDLVR	unknown
1475.84	2.45	GLFVLDGDGVLR	hypothetical protein XP688882 (E = 0.32; D. rerio)
1208.53	2.32	VFLENVLR	predicted similar to germinal histone H4 gene (E = 2.7; D. rerio)
1544.74	2.28	DNLAGGLTQPALR	oligosaccharyl transferase (E = 1.9; D. rerio)
1205.53	2.22	EELQLAQR	CLCN7 protein (E = 3.6; D. rerio)
1030.56	2.05	L(MV,TE)SLPR	unknown
1353.66	2.00	DTpSTYTE	unknown
1125.46	-2.00	SVLC(FE,YL)R	unknown
975.32	-2.00	FYAFGR	eukaryotic translation elongation factor 2-like (E = 8.8; D. rerio)
1028.52	-2.06	YNQLLR	enolase 1α (E = 8.8; D. rerio)
1011.42	-2.07	VFFDLR	unknown
1337.77	-2.12	CVNQQEFVR	hypothetical protein MGC73211 (E = 0.16; D. rerio)
992.45	-2.18	FQL(TL)R	unknown
1562.76	-2.30	SQFEGLVADLLR	heat shock protein 9B (E = 0.017; D. rerio)
1010.52	-2.31	LTGMAFR	GAPDH (E = 1.8; D. rerio)
1327.60	-2.34	VFDALMNFR	eukaryotic translation elongation factor 2-like (E = 0.93; D. rerio)
980.38	-2.58	GLCFLR	unknown
1326.64	-2.71	DFEPTH(NP)R	unknown
1636.84	-2.74	LLTVLNQHLNTR	eukaryotic translation elongation factor 1 gamma (E =0.005; D. rerio)
1180.76	**-2.83**	**FFTYHVR**	**predicted similar to complement C1q-like protein 3 precursor (gliacolin) (E = 4.2; D. rerio)**
1281.72	**-3.86**	**WDDFLCLR**	**crumbs-like protein 2b (E = 6.5; D. rerio)**
1554.90	**-4.74**	**(AS,GT)pTDLQNELSQLR**	**60S ribosomal protein L35 (E = 1.9; D. rerio)**
1172.83	**-5.21**	**LL(GE,W)PRR**	**unknown**
1690.64	**-5.41**	**TpSAAGQPSLPGGVPR**	**predicted similar rab11 binding protein (E = 1.4; D. rerio)**
1280.69	**-6.80**	**VFELLTSTR**	**proteasome activator subunit 1 (E = 2.3; D. rerio)**

Putative hypoxia biomarkers: medaka cultured cells

Protein	Hyp/Cntl	Implicated function (based upon hypoxia literature)
similar to mKIAA0821 (Danio)	11.6	unknown.
triosephosphate isomerase	5.77	induction regulated by HIF-1 pathway.
forkhead FKHR protein	3.96	undergoes changes in expression and/or phosphorylation state during intermittent hypoxia.
sequestosome 1	3.89	polyubiquitin-binding protein involved in ubiquitin proteasome degradation.
heat shock protein 70	2.85	upregulated in hypoxic mammalian heart, reduces brain injury under hypoxia.
RING finger protein	2.48	involved in ubiquitinylation pathway under hypoxia.
lactate dehydrogenase	2.00	known to be transcriptionally regulated by HIF-1.
histone H4	-2.13	histone acetylation/methylation state known to be involved in HIF-1a expression versus HIF-1a degradation via proteasome degradation pathway.
eEF2/eEF1γ	-2.36	suppressed and/or phosphorylated during hypoxia.
similar rab11 binding protein	-5.41	modulated in response to hypoxia in carcinoma cells .

Medaka liver: peptide sequences and BLAST results

m/z	Hyp/Norm	Sequence	Best BLAST match
1918.992	6.88	NDPLMQYVLNHSLR	similar to catechol-O-methyltransferase domain 1 (E = 0.002; D. rerio) unnamed protein product (E = 0.0002; T. nigriviridis)
2021.997	5.06	D(QM)DNPHLASASPHQR	predicted similar to α-fetoprotein enhancer ; AT-binding transcription factor (E = 0.24; D. rerio)
872.432	4.81	VFLHR	unknown
1428.647	4.36	TNYDNYYTR	predicted similar protocadherin 17 (E = 3.0; D. rerio) Src family associated protein 2 (E = 1.4; T. rubripes)
1586.909	4.05	HWFGPELR	unknown
		FMCYFHFLR	
		(NL,QV)PCYFHFLR	
1236.623	3.87	MFLPLLQR	similar silencer-associated factor (E = 2.7; D. rerio)
996.432	3.70	LVFELR	unknown
1408.784	3.05	DSTLLMQLLR	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (E = 0.041; D. rerio)
1647.016	2.82	LDAMHGVVGPYLR	phosphoglucomutase 1 (E = 0.0003; D. rerio)
1491.009	2.82	GNFV(N,L)DDGVNR	unknown
1867.992	2.58	ALDQLDDTPYQCGR	similar spag8-predicted protein (E = 3.3; D. rerio) LOC446267 protein (E = 0.46; X. laevis)
1451.837	2.47	VL(LP)FLSQYR	reverse transcriptase (E = 0.27; T. rubripes)
1336.624	-2.50	YQSLLNDLR	similar to SMC1β protein (E = 2.3; D. rerio)
1347.650	-2.63	GYSFTTTAER	β actin (E = 0.007; D. rerio)
1160.541	-3.23	AVFPSLVGR	β actin (E = 1.3; D. rerio)
1554.918	-3.24	EXX(E,V)EQDSHGR	unknown
1598.966	-5.88	PVLDDWMDYWNDR	sulfatase FP1c (E = 0.0002; D. rerio)
1220.717	-7.14	FQTLF(FP, LM)R	unknown

Putative hypoxia biomarkers: medaka liver

Protein	Hyp/Cntl	Implicated function (based upon hypoxia literature)
similar catechol-O-methyltransferase	6.88	activity modulated under hypoxia in mammalian brain and heart muscle tissue.
similar α-fetoprotein (afp) enhancer protein	5.06	afp modulated in human hepatic tissue under hypoxia through HIF-1 pathway.
tyrosine/tryptophan monooxygenase	3.05	tyrosine/tryptophan hydroxylases are known hypoxia-regulated genes.
phosphoglucomutase	2.82	unknown, but possibly controlled through HIF regulation of glycolytic enzymes.
β actin	-2.63	likely related to energy-conservation and/or cytoskeletal reorganization under hypoxic conditions.
sulfatase	-5.88	SULF1 known to regulate cell growth signaling in hepatocellular carcinoma (HCC); also known to modulate histone H4 acetylation in HCC: HIF-1 target gene BNIP3 known to be silenced by histone deacetylation and methylation; histone acetylation state known to be involved in modulation of hypoxia-inducible factor activation).

geLC Conclusions

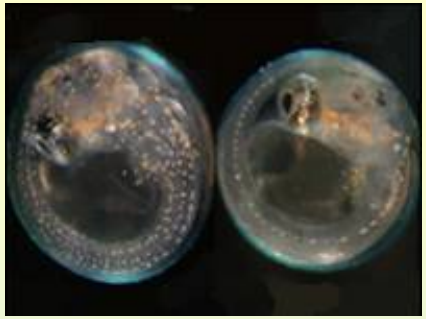
- Identified 16 putative hypoxia-related proteins (11 up, 5 down) in medaka cultured cells and liver by $^{16}O/O^{18}$-SPITC labeling and geLC-MALDI-PSD. Inclusion of DIGE-MALDI results brings total to 21 putative hypoxia biomarkers (16 up, 5 down).

- The proteins detected have also been shown to be hypoxia-regulated in other species.

- Work is currently underway to confirm the identities and fold-changes by immunochemical and molecular biology methods.

http://www7a.biglobe.ne.jp/~redcherry/kidstoppage.htm

Conclusions and Future Plans



DIGE

- 10 Brain proteins differentially expressed upon hypoxia exposure
  - Tentative protein identification
    - Succinate dehyrogenase, aldolase, carbonic anhydrase, calbindin, glutathione-S-transferase, hemoglobin
- 12 Fin proteins differentially expressed upon hypoxia exposure

geLC

- 16 putative hypoxia-related proteins (11 up, 5 down) in medaka cultured cells and liver by $^{16}O/O^{18}$-SPITC labeling and geLC-MALDI-PSD

Future plans

- Do MALDI analyses on Fin proteins
- Continue analyses on other tissues
- Confirm biomarker identifications through western blot and/or real-time PCR

Acknowledgments

- Dr. Zhenlin Ju
- Leon Oehlers
- Xiphophorus Genetic Stock Center

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Questions?