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Proteomics analysis of regenerating amphibian limbs: changes during the onset of regeneration

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ABSTRACT During amphibian epimorphic limb regeneration, local injury produces metabolic changes that lead to cellular dedifferentiation and formation of a blastema, but few details of these changes have been elucidated. Here we report the first global proteomic analysis of epimorphic regeneration comparing the profiles of abundant proteins in larval limbs of the anuran *Xenopus laevis* (stage 53) at the time of amputation (0dPA) and 3 days post-amputation when the regeneration blastema is developing (3dPA). We identified and quantified 1517 peptides, of which 1067 were identified with high peptide ID confidence. Of these 1067 proteins, 489 showed significant changes in quantity between the two groups. Taking into account identical peptides whose fold changes were within 20%, and not including peptides whose fold changes were below the observed fold changes of peptides for the internal standard (chicken lysozyme), we were able to identify 145 peptides elevated in 3dPA relative to 0dPA and 220 peptides in 0dPA relative to 3dPA. In this report, we focus on those proteins that were elevated in the 3dPA tissue relative to 0dPA. In this class were members of the annexin family (e.g. ANXA1, ANXA2, ANXA5) and the ANXA2-binding partner S100A10, which have important immunoregulatory roles in other systems and were also shown to be differentially expressed in stage 53 and 57 3dPA and 5dPA blastemas in our previous microarray studies. Besides elucidating the possible modulation of inflammation during amphibian limb regeneration, our proteomic study also provides insight into dedifferentiation by revealing up-regulation of proteins known to characterize many stem cells.

KEY WORDS: *annexin, inflammation, limb, regeneration, Xenopus*

Introduction

Among tetrapods, only urodeles such as salamanders and larval anurans have the ability to fully regenerate amputated limbs. The initial phase of regeneration includes wound closure by epidermal migration to form a wound epidermis and the reaction of deeper limb tissues to the amputation trauma. This reaction leads to the thorough remodeling of the tissues' extracellular matrices (ECM). In late larval anuran or adult urodele limbs this occurs concomitantly with histolysis, tissue and cellular dedifferentiation, and reversion of the cells to a mesenchymal state (Stocum, 2006). The mesenchymal cells proliferate to form the regeneration blastema out of which the missing portion of the limb develops in a process of epimorphic regeneration. Proliferation in the limb stump requires signaling from the apical wound epithelium and trophic factors from regenerating axons, but ECM breakdown, dedifferentiation, and renewed cell cycling all begin during the post-injury period of inflammation even in the absence of the wound epithelium and nerves (Tassava and Loyd, 1977; Tassava and Mescher, 1975).

Few studies of amphibian limb or fish fin regeneration have investigated details of the limb stump microenvironment in which ECM remodeling and cellular dedifferentiation are initiated. Gene expression during regeneration in larval limbs of *Xenopus* has been examined by subtractive hybridization (King *et al.*, 2003) and by microarray analysis (Grow *et al.*, 2006), and tail regenera-

Abbreviations used in this paper: ANXA, annexin; dPA, days post-amputation; ECM, extracellular matrix; IL-1, interleukin-1; LC/MS, liquid chromatography/mass spectroscopy; SOCS, suppressor of cytokine signaling; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha.

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tion in larval *Xenopus* has been the subject of similar molecular screens (Ishino *et al.*, 2003; Tazaki *et al.*, 2005). These and other studies have identified a large number of inflammatory and immune-related genes active during the early phase of regeneration. Expression of several genes with well-known roles in modulation of inflammation and immunity, including *suppressor of cytokine signaling-3* (*SOCS3*), *stress-inducible tumor rejection antigen gp96* (*TRA1*), *fibrinogen-like protein 2* (*FGL2*), *myeloid differentiation factor 88* (*MyD88*), and *complement 3* (*C3*), was found by qPCR to be strongly up-regulated locally within one day after amputation in *Xenopus* (Grow *et al.*, 2006).

Possible roles for other specific components of innate immunity have also been uncovered during both limb and lens regeneration in adult newts. Both systems involve local synthesis of complement C3 and C5 (Kimura *et al.*, 2003). Thrombin and tissue factor (which is involved in conversion of prothrombin to thrombin) have been shown to be important for dedifferentiation of muscle fibers in regenerating limbs (Kumar *et al.*, 2004) and pigmented epithelial cells in lens regeneration (Imokawa *et al.*, 2004). These and other aspects of immune involvement in regeneration have recently been reviewed (Godwin and Brockes, 2006; Mescher and Neff, 2006).

As another approach to investigate cellular activities in amputated limbs during the early phase of regeneration, we have undertaken proteomic analyses of larval *Xenopus* hindlimb stumps at premetamorphic stage 53 when essentially complete regeneration is possible. Proteomic data obtained from these regenerating limbs at this developmental stage can be compared directly with our previous molecular screens of limb regeneration. The major goals of the study were to examine changes in the proteome of the developing limb that are triggered by amputation and to gain further insight into the role of inflammatory activity occurring locally in the limb stump three days after amputation.

The results extend findings from our screens of gene activity during limb regeneration (King *et al.*, 2003) identifying immune-related genes expressed locally as a regeneration blastema is formed (Grow *et al.*, 2006). Among the proteins we found to be most elevated in concentration after amputation are several members of the annexin family of highly conserved Ca^{2+} and phospholipid binding proteins important for many aspects of membrane organization and membrane traffic. The protein with one of the highest observed fold-changes (FC), annexin-1 (ANXA1), which mediates the anti-inflammatory activity of glucocorticoids (Wu *et al.*, 1995) plays a major role in the resolution of inflammation (Perretti and Flower, 2004; Scannell *et al.*, 2007). Since ANXA1 is upregulated in response to spinal cord injury (Liu *et al.*, 2007) and in the regenerating salamander spinal cord (Monaghan *et al.*, 2007) where it may play neuroprotective and anti-inflammatory roles (Solito *et al.*, 2008) it has great interest for the field of regenerative biology. Other abundant proteins found to be strongly up-regulated after amputation include specific keratins with roles in cytoprotection and growth regulation during wound closure, some of which have been previously implicated in the control of blastema growth. The results highlight the importance of local inflammation and its resolution during the initial phase of limb regeneration, when dedifferentiation and early events of blastema formation are underway.

TABLE 1

PROTEIN CATEGORY, NUMBER OF SIGNIFICANT CHANGES AND EXHIBITED VARIABILITY (COEFFICIENT OF VARIATION) FOR EACH PROTEIN CATEGORY

Protein Category	Peptide ID Confidence	Multiple Sequences	Number of Proteins	Number Significant Changes	Median %CV replicate	Median %CV rep + sample
1	HIGH	YES	555	310	8.55	9.68
2	HIGH	NO	512	179	14.54	15.81
3	LOW	YES	27	7	17.86	19.08
4	LOW	NO	423	114	20.68	24.61
Overall			**1517**	**610**	**12.62**	**14.44**

Proteins with best peptide having a confidence between 90-100% are assigned to the 'HIGH' category. Proteins with best peptide having a confidence between 75-89% are assigned to the 'LOW' category. The replicate median % coefficient of variation (%CV) is the standard deviation divided by the mean on a % scale.

Results

Statistically significant proteins in regenerating blastemas

In order to identify proteins whose levels are higher in the regeneration-competent limb in response to amputation, we carried out mid-zeugopodial amputations of stage 53 hindlimbs, a stage at which gene expression during regeneration has been analyzed previously (King *et al.*, 2003; Grow *et al.*, 2006). We collected tissue at the site of amputation either immediately (0dPA) or 3-days post-amputation (3dPA) and subjected the samples to LC/MS analysis. By comparing the proteomes at these two times we were able to identify and quantify 1517 differentially expressed peptides. These proteins were then classified according to the confidence of the peptide identification as shown in Table 1.

A total of 489 peptides identified with a high confidence value (categories 1 and 2) were found to have statistically significant differences in expression at the two time points. Although we present data sets encompassing proteins higher in the 3dPA tissue and 0dPA tissue, we focus on the proteins with higher levels in 3dPA tissue relative to 0dPA. Eliminating those with FC lower than that of the internal standard, chicken lysozyme, we were left with a total of 145 differentially expressed peptides listed in Table 2. Of the 145 individual peptides listed in Table 2, 136 have identifiable protein names and are listed first, sorted according to peptide identification category (i.e. category 1 then 2) followed by the peptides whose sequences were identified only as hypothetical, unknown or novel. Each peptide is listed with its annotated protein name, UniGene identifier and the accession number associated with full-length mRNA sequences. Although our focus was on proteins elevated in response to amputation and the onset of regeneration, shown in Table 3 are the 220 peptides identified with high confidence whose levels were higher in the limb amputation site at the time of amputation (0dPA) relative to 3dPA.

Several identical (as well as different) peptides representing the same protein were identified in our screen and these peptides are listed independently in Tables 2 and 3. The peptides were listed independently because either the FC values for the identified peptides were different or the peptide ID confidence values placed the peptides in different categories (i.e. category 1 versus category 2). Higgs *et al.* (2005, 2007) suggest that ~10% reproducibility in quantitative measurements is possible using label-

TABLE 2

PROTEINS EXPRESSED AT ELEVATED LEVELS IN 3dPA RELATIVE TO 0dPA

Protein Identification by Peptide(s)	Peptide	UniGene ID	Accession	FC	Category	Biological Process
annexin 1	GVDEGTIIDILTK	ANXA1	BC053786	1.763	1	CC,ST
keratin 12	SLEEQLLQIR	KRT12	BC108476	1.621	1	CG,M
larval alpha globin	LFLSYPQTK	HBA	BC054259	1.619	1	T
granulin	DVQCDDMYSCPDGQTCCR	GRN	BC048224	1.578	1	CC,ST
apolipoprotein-A1	LDTFGTNAMNLR	APOA1	BC077663	1.504	1	T
major vault protein	EAIPLDENEGIYVR	MVP	BC057708	1.446	1	CG,M
ferritin heavy chain	DEWSNTLEAMQAALQLEK	FTH1	BC044961	1.428	1	T
transthyretin	GIPAANLLVNVFR	TTR	AB026996	1.425	1	T
phosphoserine aminotransferase 1	VLFLQGGGSGQFSAIPLNLIGLK	PSAT1	NM_001016582	1.410	1	M,EP
myeloperoxidase, peroxidase 2'	VGELLACLIGDQFR	MPO-A	AY069942	1.387	1	M,EP
alpha globin larval-8	IAPQASAIGAEALER	HBA-8	BC135231	1.386	1	T
fibrinogen gamma	IELEDWSNQK	FGG	BC054185	1.378	1	PM
polysomal ribonuclease 1	DWLPLLLGSEMAAVLPAYR	PMR-1	U68724	1.377	1	PM
transferrin, lactotransferrin	SNNEPYYNYAGAFK	TLTF	BC054950	1.355	1	T
annexin 5	VNDSLVEQDAQDLFK	ANXA5	BC082506	1.339	1	CC,ST
larval keratin	WTLLQEQGGQVK	LK-A	AB045599	1.339	1	CG,M
transketolase	AVPTATVFYPSDAVSTEK	TKT	BC093574	1.332	1	M,EP
B fibrinopeptide	GICDMPGEFWLGNEK	FGB	BC084842	1.329	1	PM
serine (or cysteine) proteinase inhibitor member 1d	DVFSDLADLTGIAASR	SERPINA1D	BC054235	1.324	1	PM
keratin B1/B2	DAELWFNQK	KRTB1/KRTB2	X04805	1.322	1	UNK
thioredoxin	VDVDNASDVAQLCGVR	TXN	BC084818	1.321	1	M,EP
interferon regulatory factor 2 binding protein 2	MPWAMIWDFTEPVCR	IRF2BP2	BC081137	1.309	1	RN
ferritin	LGVPQNGMGEYLFDK	FTN	BC061303	1.282	1	T
ATPase, Na+/K+ transporting, alpha 3 polypeptide	DVAGDASESALLK	ATP1A3	BC043743	1.274	1	T
profilin 2	GTPSVWACTPGGVFSNITPAEINALVSPNR	PFN2	BC053770	1.273	1	CG,M
lymphocyte cytosolic protein 1	ISTSMPVLDLIDAIQPGCINYDLLK	LCP1	BC056055	1.267	1	CC,ST
alpha globin larval-6	AAIASLWGK	HBA-6	DQ224416	1.265	1	T
fibrinogen A alpha polypeptide	NWPICSDEDWGPK	FGA	BC041754	1.264	1	PM
vimentin-1/vimentin-4	QIQTLTCEIDAMK	VIM1/VIM4	BC045233	1.260	1	CG,M
similar to Rho GDP-dissociation inhibitor 2	SLLGDGPVVADPSAPNVTVTR	ARHGDIB	BC087424	1.259	1	CC,ST
member RAS oncogene family, Rab43	LQIWDTAGQER	RAB43	BC097569	1.258	1	CC,ST
beta globin	LLVVYPWTQR	HBB	BC078515	1.254	1	T
serum albumin B precursor	SCFTALGPDEDYVPPPVTDDTFHFDDK	ALBB	BC081223	1.252	1	T
beta globin larva-1	LGAAFTPQVQAAWEK	HBB-1	BC078582	1.251	1	T
keratin complex 2, basic, gene 5	CNIDPLFEAYISSLR	KRT2-5	BC121489	1.249	1	CG,M
prohibitin	FDAGELITQR	PHB	BC061380	1.243	1	CC,ST
transgelin 2	YGIPASDLFQTVDLWEGK	TAGLN2	BC072141	1.238	1	UNK
voltage-dependent anion channel 3	LSLDTTFVPNTGK	VDAC3	BC071123	1.234	1	T
prosaposin (variant Gaucher disease)	LVSDVQDALR	PSAP	NM_001017285	1.220	1	CC,ST
similar to apolipoprotein A-I	DAVSQLETSDLGK	APOA1	BC041498	1.213	1	T
fibronectin	YNVNVYQITEEGEK	FN1	BC072841	1.213	1	CG,M
annexin 2, annexin II type 1	GDLENAFLNLVQCIQNKPLYFADR	ANXA2	BC042238	1.206	1	CC,ST
cytoplasmic beta actin	DLYANTVLSGGTTMYPGIADR	ACTB	BC084121	1.190	1	CG,M
collagen, type I, alpha 1	GPPGPSGSPGPQGFQGPPGEPGEPGSSGAMGPR	COL1A1	BC049829	1.188	1	CG,M
non-muscle myosin II heavy chain	ELESQIGELQEDLESER	NMMHC	BC047253	1.184	1	CG,M
similar to Staphylococcal nuclease domain containing 1	DTSGENIAESLVAEGLASR	SND1	BC063211	1.182	1	RN
annexin 1	GLGTDEDTLIEILASR	ANXA1	BC053786	1.176	1	CC,ST
annexin 2, annexin II type 1	GALSGNLETVMLGLIK	ANXA2	BC042238	1.174	1	CC,ST
transforming growth factor beta-induced 68kDa	QYTLLAPTNEAFEK	TGFB1i1	BC121403	1.151	1	CC,ST
keratin complex 2, basic, gene 5	LESGFQNLSIQTK	KRT2-5	BC121489	1.148	1	CG,M
collagen, type I, alpha 2	GTPGESGAAGPFGPLGPR	COL1A2	BC049829	1.147	1	CG,M
complement component 1 q subcomponent binding protein	AEENEPELVSTPNFVVEVLK	C1QBP	BC056846	1.146	1	IR
NM23/nucleoside diphosphate kinase	FQQASQDLLR	NM23NDK-A	BC079795	1.139	1	M,EP
fetuin B	TEYIQFPEVASHLPTCPLIIEEK	FETUB	BC078490	1.139	1	IR
glutathione-S-transferase	QMASEPLPPELLEFLK	GST	BC072203	1.137	1	M,EP
larval keratin	AGLEASLADTEGR	LK-A	AB045599	1.136	1	CG,M
transforming growth factor beta-induced 68kDa	GCPAALPLSNIYETLGIVGAATTQLYSDR	TGFB1i1	BC121403	1.134	1	CC,ST
non-muscle myosin II heavy chain	IVGLDQVAGMGDTALPGAFK	NMMHC	BC047253	1.128	1	CG,M
collagen, type I, alpha 1	SAGISMPGPMGPMGPR	COL1A1	BC049829	1.126	1	CG,M
putative cathepsin L	LISLSEQNLVDCSR	CTSL	BC060335	1.123	1	PM
ribosomal protein S19	ELAPYDENWFYTR	RPS19	BC056505	1.121	1	PM

TABLE 2 (continued)

PROTEINS EXPRESSED AT ELEVATED LEVELS IN 3dPA RELATIVE TO 0dPA

Protein Identification by Peptide(s)	Peptide	UniGene ID	Accession	FC	Category	Biological Process
myosin regulatory light chain	ATSNVFAMFDQSQIQEFK	MRLC2	CR760144	1.114	1	CG,M
moesin	IGFPWSEIR	MSN	BC121565	1.111	1	CG,M
annexin 1	TPAEFDAYELK	ANXA1	BC053786	1.099	1	CC,ST
similar to myosin light chain 1, skeletal muscle isoform A1 catalytic	TLEFEQFLPMLQAIAK	MLC1F/3F	BC092347	1.099	1	CG,M
tropomyosin	IQLVEEELDR	TPM	BC070998	1.093	1	CG,M
glyceraldehyde-3-phosphate dehydrogenase type B	VPVPNVSVVDLTCR	GAPDHB	AF549496	1.090	1	M,EP
lumican	IDLPQDMYSCLR	LUM	BC054282	1.087	1	CG,M
phosphoserine aminotransferase 1	DVLNFGAGPAK	PSAT1	NM_001016582	2.630	2	M,EP
keratin	TVIEEVVDGK	KRT1-2	BC045031	1.822	2	CG,M
SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily d, member 1	TQMNSFLLSTASQQEIAALDNK	SMARCD1	CR926205	1.760	2	RN
procyclic acid repeat protein	EELGFLPSYSASQLK	PARP	BC110778	1.738	2	UNK
solute carrier family 26, member 6	VDTLIELKK	SLC26A6	BC097666	1.682	2	T
alkylated DNA repair protein alkB homolog 5	SVLLPKQRR	ALKBH5	BC073226	1.656	2	UNK
novel protein similar to PTPRF interacting protein, binding protein 2	VTSQLHHLSIK	PPFIBP2	CR926392	1.610	2	CG,M
slingshot-related protein	EETERIIKLKLRDI	SSH	BC080117	1.604	2	CG,M
calcium-binding protein p26olf	DGCADTMTYQEFEDFMK	LOC443554	AB063625	1.603	2	UNK
ATPase, H+ transporting, V0 subunit B	LAIVSQNLQK	ATP6V0B	BC090362	1.566	2	T
neurofilament 3	EYQDLLNVK	NEF3	BC118832	1.539	2	CG,M
beta globin	LLVVYPWTQR	HBB	BC078515	1.524	2	T
amyloid-beta-like protein A precursor	QLSGKDIITDVK	APLP2 A	AJ608932	1.503	2	CC,ST
aldolase A	GVVPLAGTNGETTTQGLDGLSER	ALDOA	BC106622	1.503	2	M,EP
manganese superoxide dismutase	MRCVPALAYSFCK	SOD2	AY362041	1.479	2	CP
glucokinase	HEDIDKGILLNWTK	GCK	X93494	1.416	2	M,EP
keratin 17	YCMQLSQIQGLIGNVEAQLADLR	KRT17	BC074309	1.410	2	CG,M
DNA repair and recombination protein 54	MTAEPMSESK	RAD54	BC094402	1.407	2	RN
fibrinogen A alpha polypeptide	NWPICSDEDWGPK	FGA	BC041754	1.392	2	PM
cofilin	EDLTLQQLAEK	CFL1	BC045044	1.389	2	CG,M
IkappaB kinase complex-associated protein	ANLSLLGGAKK	IKBKAP	BC110776	1.369	2	CC,ST
galectin VIIa	VPYDLPLPSGVVPR	LGALS7	AB080020	1.358	2	CG,M
integrin alpha V subunit	YETDGPMDCTSDVEINPLNVK	ITGAV	U92006	1.357	2	CC,ST
elastase 2	VNQVFENGFNPLTLENDIVILK	ELA2	BC106661	1.351	2	PM
SP22	DVMLCPDTSLEEAR	PARK7	AF394958	1.336	2	RN
aldehyde dehydrogenase 7 member A1	CEGGTVVCGGK	ALDH7A1	NM_001016377	1.308	2	M,EP
proteasome (prosome, macropain) 26S subunit, non-ATPase, 12	DPNDLLNDWSQK	PSMD12	CR848271	1.297	2	PM
phospholipase D	VAVNPPDSPIR	PLD3	BC059981	1.295	2	M,EP
annexin 7	AAPNFDALSDAEK	ANXA7	U16365	1.293	2	T
glucose-6-phosphate isomerase	ELQASGLTGEALDK	GPI	BC073315	1.287	2	M,EP
ribosomal protein L19	VWLDPNETNEIANANSR	RPL19	BC041546	1.286	2	PM
kinesin light chain 4	LVMQGLEALR	KLC4	BC043636	1.277	2	T
ATP-dependent chromatin remodeling DEXH-box protein, BRG1	AIEEGTLEEIEEEVR	SMARCA4	AY762376	1.277	2	RN
ribosomal protein S11	CPFTGNVSIR	RPS11	X78805	1.269	2	PM
calpactin I (annexin II) light chain p11 subunit	LLDSEFSEFLK	S100A10	BC088081	1.263	2	CC,ST
calpain 2	NFPETFWMNPQYMIK	CAPN2	BC063733	1.260	2	PM
phosphatidylserine decarboxylase	AVYSRAPTR	PISD	BC074595	1.260	2	M,EP
gelatinase B, matrixmetalloproteinase 9	ILNYSPDLDPEVIDDAFAR	MMP9	AF072455	1.251	2	PM
dipeptidylpeptidase 3	TVADQMYSLEPAER	DPP3	BC060495	1.250	2	PM
similar to matrix metalloproteinase 13 (collagenase 3)	AFGVWSNVTPLQFTK	MMP13	BC046939	1.250	2	PM
similar to complement component 1, q subcomponent binding protein	EVSFQPTGDTEWK	C1QBP	BC056846	1.249	2	IR
ubiquitin specific protease 33	CLMDVLHEELK	UCHL1	NM_00106228	1.237	2	PM
c-met/hepatocyte growth factor receptor	IYVLNENLTK	MET	AB027411	1.231	2	CC,ST
non-muscle myosin smooth muscle	NMDPLNDNVTALLNQSSDK	NMMHC	BC084652	1.229	2	CG,M
trypsin	INQVFENGFNPMNLENDIVILK	PRSS1	BC108835	1.227	2	PM
similar to cytochrome P450 3A70	TVLSPTFTSGKLK	CYP3A70	BC084827	1.225	2	M,EP
enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	AEFGQPEILLGTIPGAGGTQR	ECHS1	BC074535	1.208	2	M.EP
prosaposin	LVSDVQDALR	PSAP	NM_001017285	1.206	2	CC,ST
programmed cell death 4	MEIETPSDINANVESDSELSGVEQNR	PDCD4	BC082619	1.202	2	A

TABLE 2 (continued)

PROTEINS EXPRESSED AT ELEVATED LEVELS IN 3dPA RELATIVE TO 0dPA

Protein Identification by Peptide(s)	Peptide	UniGene ID	Accession	FC	Category	Biological Process
DEAH (Asp-Glu-Ala-His) box polypeptide 9	ELDALDSNDELTPLGR	DHX9	CR760714	1.196	2	RN
lamin A/C	MQQQLDEYQELLDIK	LMNA	NM_001083354	1.192	2	CG,M
similar to cathepsin B	ILGWGVENGTPYWLCANSWNTDWGDNGFFK	CTSB	BC063365	1.187	2	PM
adult keratin	MSYSCVK	AK-B	AB045601	1.172	2	CG,M
prosaposin	TGDICNDCTTLISDVQDALR	PSAP	NM_001017285	1.171	2	CC,ST
suppressor of cytokine signaling-6	YLLSLSFR	SOCS6	BC077367	1.154	2	CC,ST
guanine nucleotide-binding protein alpha-14 subunit	VPTTGIIEYPFDLENIIFR	GNA14	BC080940	1.149	2	CC,ST
methionyl aminopeptidase 2	SQTDPPSIPISELYPSGVFPK	METAP2	NM_00106115	1.146	2	PM
transaldolase 1	NLGGSEEEQINNIMDK	TALDO1	BC084118	1.144	2	M,EP
copine	DIVQFVPFR	CPNE1	BC106238	1.144	2	T
junction plakoglobin	LNTIPLFVQLLYSPVENIQR	JUP	BC094116	1.141	2	CC,ST
collagen VI	QSVAFPLAFDLTEVSQAIEK	COL6A3	BC089181	1.137	2	CG,M
member RAS oncogene family, Rab33B	IQLWDTAGQER	RAB33B	BC081311	1.121	2	CC,ST
Ras-related protein Rab	LQLWDTAGQER	RAB1A	BC118789	1.121	2	CC,ST
ribosomal protein L13	GFSLEELK	RPL13	CR760720	1.119	2	PM
member RAS oncogene family, Rab43	LQIWDTAGQER	RAB43	BC097569	1.118	2	CC,ST
suppressor of Ty, domain containing 1	QPSSSGAVRPSSGPPTGATPK	SPT2D1	BC121680	1.104	2	RN
Rab3 GTPase-activating protein catalytic subunit	ESPLNNDVLNAILFFLF	RAB3GAP1	BC081089	1.101	2	CC,ST
similar to adenylosuccinate synthetase	VVDLLAQDADIVCR	ADSS2	CR761467	1.100	2	M,EP
Novel, Hypothetic, or Unknown Proteins						
	QNHVVVLDSR	MGC82459	BC077467	1.721	2	
	MQSNSSLVPEK	**MGC81344**	BC076732	1.645	2	
	ISVLESELASLR	MGC82812	BC073542	1.484	2	
	QGILNILAAR	MGC80123	BC072800	1.323	2	
	ILLSVGFSR	LOC548906	NM_00106152	1.300	2	
	VSFMVTPSLFVDPLYGR	LOC398539	BC076719	1.249	1	
	IGYNFDPISR	MGC:115035	BC094478	1.204	2	
	SKPSLPPGLSETDASTGK	LOC549028	NM_00106274	1.197	2	
	DETWVNVSSPPLLEDPLLK	MGC:116540	BC099345	1.181	2	

Protein names are those determined from the peptide of highest confidence with the peptide sequence shown. The proteins are sorted by FC with category 1 peptides listed first followed by category 2. Only proteins with FC above that of the internal standard are listed. Accession numbers are those sequences that identified the peptide of highest confidence. Function refers to "Biological Process" as defined by the Human Protein Reference Database (HPRD). Protein names (or UniGene ID for unknowns) listed in bold type are those whose encoding gene was also found expressed at higher levels in stage 53 3dPA versus 1dPA (unpublished). Abbreviations: CG,M = cell growth and/or maintenance; CC,ST = cell communication, signal transduction; T = transport; PM = protein metabolism; M,EP = metabolism, energy pathways; RN = regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism; CP = cell proliferation, anti-apoptosis, cell growth and/or maintenance; A = apoptosis; IR = immune response; UNK = unknown; FC = fold change.

free proteomics. However, as our cut-off for determining whether or not to include multiple examples of the same peptide in Tables 2 and 3, we used the more conservative value of 20% reproducibility in the FC differences between two or more instances of the same peptide.

A relatively large disagreement in fold changes for the same peptide in replicate measurements might be due to a number of subtle phenomena. For example, variable post-translational modifications such as oxidation, signal levels near the mass spectrometer's limit of detection, and the presence of co-eluting peptides with strong signals whose intensity or retention time changes, could all contribute to inconsistent fold changes across replicates. Taking into account the fact that different peptides representing the same protein (but with different FC values) were identified, Table 2 contains 122 unique annotated proteins along with the 9 unknowns, and Table 3 contains 192 unique annotated proteins and 10 unknowns.

In our previous array screens [(Grow *et al.*, 2006) and unpublished 3dPA data] we identified a much larger number of differentially expressed genes between two pairs of limb tissues. However, we believe that the total number of protein differences we found in the present study is highly relevant for several reasons. Our complex proteomic analysis was carried out using tryptic digests from whole limb stump or blastema tissue. There was no fractionation of the samples prior to injection of the tryptic peptides into the LC column, yet we were able to identify 1517 differentially expressed proteins. Since subcellular fractionation prior to proteomic analysis can greatly enhance the identification of low abundance proteins (Mitulovic and Mechtler, 2006; Brown *et al.*, 2007), detection of such proteins is likely to be limiting in our assay. Indeed many proteins one might have expected to show significant FCs based on our previous array screens were not identified. In addition, we have analyzed the differences in gene expression between 1dPA and 3dPA in stage 53 limbs (unpublished), and among those with higher expression at 3dPA are 30 genes encoding proteins listed in Table 2 (e.g. *ANXA1*, *ITGVAV*, *IRF2BP2*, *SMARCA4*, and *PSAP*). Lastly, as discussed below several proteins identified in our study have been shown to be differentially expressed in other models of limb regeneration.

The proteins found more highly concentrated in limb tissues following amputation were functionally classified by their biological process using categories defined by the Human Protein Reference Database [www.hprd.org; ref. (Peri *et al.*, 2003)]. As indicated in Table 2 most of the proteins are involved in cell growth

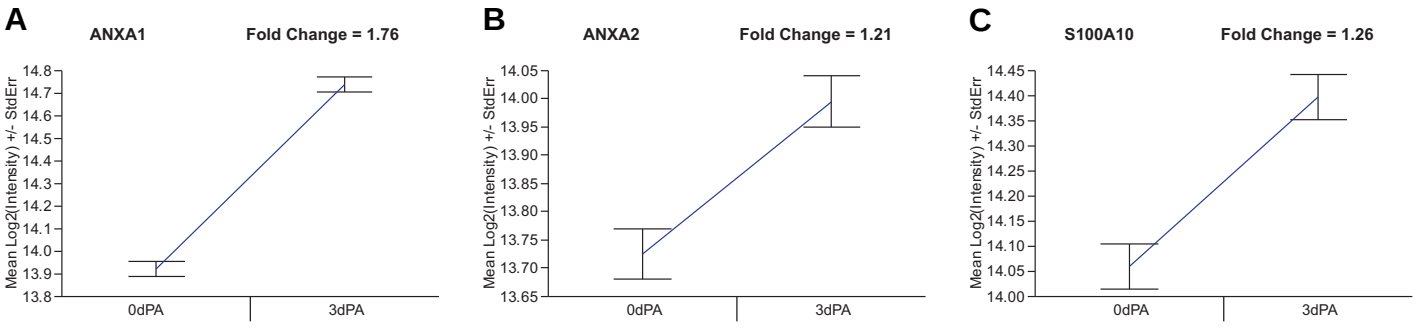


Fig. 1. Peptide fold-changes. *Mean log2 intensity comparisons for ANXA1, ANXA2 and the ANXA2-binding partner S100A10. Relative protein expression levels at 0dPA compared to 3dPA represent the mean value determined from 10 independent injections. The intensities which are given by the area under the curve (AUC) from the extracted ion chromatography (XIC) were transformed to the log2 scale. Error bars show standard errors based on the ANOVA model.*

and maintenance, cell communication or signal transduction, transport, metabolism and energy pathways. The cell growth and maintenance proteins include specific keratins that were identified in previous immunocytochemical studies as up-regulated in adult newt limb regeneration (see Discussion). Many other abundant proteins listed in Table 2, including matrix metalloproteinases, fibronectin, type I collagen, vimentin, and non-muscle myosin have also been found to be expressed or concentrated more highly in regenerating adult urodele limbs using unrelated methods (reviewed by Geraudie and Ferretti, 1998). Our identification here of many specific proteins shown by other investigators to appear after limb amputation in other species and using completely different experimental techniques, strongly supports the validity of the proteomics approach to the understanding of the regulation of regeneration in this system.

Since stage 53 limbs consist almost entirely of proliferating epithelial and mesenchymal cells it is not surprising that a significant percentage of the proteins we identified at elevated levels in such limbs (0dPA) are members of the DNA replication process, RNA synthesis and translation (Table 3). Ribosomal proteins and translation factors represent 24% of the proteins identified by high confidence peptides. Protein families that are involved in replication, RNA synthesis and transport including replication licensing factors, histones, ribonucleoproteins and chaperones constituted the next highest percentage of identified peptides.

Amputation of stage 53 limbs interrupts the rapid cell proliferation to allow for epithelial closure of the wound and the inflammatory response which together initiate wound repair and then, by 3dPA, the events which lead to limb regeneration. Therefore, a reduced number of growth and proliferation associated proteins in the 3dPA tissue is to be expected and was in fact found. While, the peptides identified in our screen represent statistically significant differences between the two time points, it is still important to note that we did not find any peptides in 3dPA tissue that were also in 0dPA tissue (compare Tables 2 and 3).

Proteins with immune function

Microarray and other gene expression studies suggest that proteins involved in immune defense and other aspects of inflammation are particularly important in the post-amputation regenerative response of amphibian limbs (Harty *et al.*, 2003; Grow *et al.*, 2006; Mescher and Neff, 2006). Similar results are reported in other regenerating systems (Putta *et al.*, 2004; Lien *et al.*, 2006; Schebesta *et al.*, 2006), although the inflammatory process as a whole represents a poorly studied aspect in any model of epimorphic regeneration. To identify additional factors with immune-related activity among the proteins up-regulated three days after amputation, we compared our data with the results of gene expression analyses of various immune cell types, e.g. that of Abbas *et al.*, (2005) and other reports of immune function. Among the 136 up-regulated proteins, 24 were identified as having immune-related functions (Table 4). These include components of the coagulation pathway and of the complement cascade (C1QBP); proteins with antimicrobial activity (e.g. lactoferrin); markers for macrophages, neutrophils, and other leukocytes (e.g. granulin, vimentin, myeloperoxidase); as well as factors with both pro- and anti-inflammatory activity (e.g. interferon regulatory factor 2 binding protein).

Of the immune-related proteins differentially expressed in this screen we chose members of the annexin family for further analysis. Expression of annexins has been demonstrated in most molecular screens of regeneration markers in several model systems; and as discussed below, well-characterized functions of annexins have potential importance to the regeneration process. It is of interest that of 13 annexin family members four (ANXA1, ANXA2, ANXA5, and ANXA7) were significantly higher in the 3dPA blastemas compared to the 0dPA limbs. Each of these four annexins was detected at a fold change higher than the chicken lysozyme internal standard. By contrast only one annexin (ANXA6) was higher in 0dPA limbs (see Table 3).

ANXA1 and ANXA2 were each identified with multiple sequences and ANXA1 was among those proteins most strongly up-regulated 3 days after amputation. Shown in Figure 1 are the relative protein expression levels, identified at 0dPA and 3dPA, for ANXA1 and ANXA2 and S100A10 (an important dimerization partner with ANXA2). These represent the mean expression level determined from the 10 independent injections (see Methods). Protein levels are presented as the log base 2 value after quantile normalization, and the fold change for these and all proteins in Tables 2 and 3 is determined from the antilog values of expression level.

In order to compare protein differences to gene expression level differences we assessed the relative expression levels before and after amputation of *ANXA1*, *ANXA2*, and *S100A10* by quantitative RT-PCR (Figure 2). These results indicate that gene expression of all three is up-regulated 3 days after amputation. As

TABLE 3

PROTEINS EXPRESSED AT ELEVATED LEVELS IN 0dPA RELATIVE TO 3dPA

Protein Identification by Peptide(s)	Peptide	UniGene	Accession	FC	Category	Biological Process
histone H2A	AGLQFPVGR	H2A.Z12	BC044011	2.256	1	RN
ribonucleotide reductase subunit M1	DLFYAMWIPDLFMK	RRM1	BC074185	1.395	1	RN
histone H2A	AGLQFPVGR	H2A.Z12	BC044011	1.377	1	RN
proliferating cell nuclear antigen	AEDNADTVTMVFESPNQEK	PCNA	BC080365	1.328	1	DNAR
similar to histone H1.4 (histone H1b)	SGVSLAALK	H1E	X03017	1.293	1	RN
oocyte translation elongation factor 1 alpha	IGGIGTVPVGR	EEF1A	BC045083	1.282	1	PM
DNA replication licensing factor 7	YTNLFADAVQELLPQYK	MCM7	BC067307	1.281	1	RN
fascin	ISCFSPSVSPAEK	FSCN1	BC097600	1.263	1	CP
ribosomal protein S15	GVDLDQLLDMSYEQVMQLYCAR	RPS15	BC053812	1.258	1	RN
H2A histone family, member V	GDEELDSLIK	H2AFV	BC091605	1.251	1	RN
fusion protein in t(12;16) of malignant liposarcoma	AAIDWFDGK	FUS	NM_001092824	1.248	1	RL
cytosolic thyroid hormone binding protein/pyruvate kinase type M2	GDLGIEIPAEK	PKM2	BC079921	1.243	1	M,EP
DNA replication licensing factor 2	AAEGAPEEDEEMIESIENLEDMK	MCM2	BC046274	1.236	1	RN
glutamate oxaloacetate transaminase 2	NLDKEYLPIGGLAEFAR	GOT2/ASP2	BC045269	1.233	1	M,EP
RAB11B, member RAS oncogene family	VVLIGDSGVGK	RAB11B	BC041250	1.232	1	CC,ST
macrophage migration inhibitory factor	DSVPDTLLSDLTK	MIF	BC097727	1.232	1	CC,ST
heterogeneous nuclear ribonucleoprotein K	VILDLISESPVK	HNRPK	BC044711	1.223	1	RN
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1	ALAALVSQLLQFQEDAFGR	SMARCC1	BC044014	1.223	1	RN
chaperonin containing TCP1 subunit 5	CPSLEQYAMR	CCT5	BC044997	1.222	1	PM
glycine cleavage system protein H	MTVDNPSELDDLMSEDAYEK	GCSH	AL845787	1.221	1	M,EP
high density lipoprotein binding protein (vigilin)	VVTEIMQETGTR	HDLBP	BC044314	1.216	1	T
RAN, 25kDa ras-related protein	VCENIPIVLCGNK	RAN	BC074619	1.216	1	CC,ST
heterogeneous nuclear ribonucleoprotein K	GSFGDIGGPVVTTQVTIPK	HNRPK	BC044711	1.215	1	RN
small nuclear ribonucleoprotein polypeptide D2 isoform	REEEEFNTGPLSVLTQSVK	SNRPD2	CX467787	1.212	1	RN
heteronuclear ribonucleoprotein A/B	IFVGGLNPEAGEDK	HNRPAB	NM_001087020	1.212	1	RN
chaperonin containing TCP1 subunit 4	VIDPQTANSVDLR	CCT4	BC076940	1.211	1	PM
heteronuclear ribonucleoprotein A/B	GEEDAGCVADISSPLTEGVK	HNRPAB	NM_001087020	1.208	1	RN
histone 2, H2bf	AMSIMNSFVNDVFER	H2BF	CR855675	1.208	1	RN
lamina-associated protein-2 beta isoform	EMFPTEFSTPTGISASCR	LOC780742	AF048815	1.207	1	UNK
zinc finger protein 326	SQGGSSWDPSFTR	ZNF326	CR762202	1.207	1	RN
DNA replication licensing factor 3	TPMENIGLQDSLLSR	MCM3	BC089251	1.205	1	RN
translation initiation factor-5	EDLKIPDGDLGK	EIF5	BC045007	1.202	1	PM
ribosomal protein L5	RFPGYDSESK	RPL5	BC059751	1.200	1	PM
SET translocation	VEVTEFEDIK	SET	CK740651	1.199	1	RN
chaperonin containing TCP1 subunit 5	AFADALEIIPMSLAENSGMNPIQTMTEVR	CCT5	BC044997	1.197	1	PM
HMG-X protein	LGELWAEQTPK	MGC52578	BC044715	1.197	1	UNK
similar to ADP-ribosylation factor-like 6 interacting protein	WQNNLLPSR	ARL6IP	BG579961	1.197	1	RN
moderately similar to RNA-binding protein AUF1	IFVGGLSPDTPEDK	HNRPD	BC097855	1.196	1	RN
histone H3a	ALQEASEAYLVGXFEDTNLCAIHAK	H3A	DQ284418	1.196	1	RN
zygotic DNA replication licensing factor 6-B	CDFTGSLIVVPDISQLSTPGVR	MCM6b	AF031139	1.193	1	RN
heterogeneous nuclear ribonucleoprotein R	DLFEDELVPLFEK	HNRPR	CA988055	1.192	1	RN
similar to SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c2	LNPQEYLTSTACR	SMARCC2	BC136222	1.191	1	RN
high mobility group protein 2a	LGEMWNNLSDGEK	HMG2a	BC123147	1.191	1	RN
glycerol-3-phosphate dehydrogenase 1-like	LPENVVALPNLTDAVR	GPD1L	BC076683	1.189	1	UNK
TAR DNA-binding protein	TSDLIVLGLPWK	TARDBP	BC044271	1.188	1	RN
ribosomal protein L31	LYTLVTYVPVTNYK	RPL31	BC077057	1.186	1	PM
proteosome subunit beta type 1	AGGSASAMLQPLLDNQIGYK	PSMB1	NM_001086966	1.184	1	PM
weakly similar to 60S acidic ribosomal protein P2	LSSVPCGGAVSAAPASTPAAGGAAPAEK	RPLP2	BC023136	1.184	1	PM
nucleosome assembly protein 1-like 1	QLTAQMMQNPQVLAALQER	NAP1L1	NM_001087078	1.182	1	RN
moderately similar to 60S acidic ribosomal protein P1	TAGVTVEPFWPSLFAK	RPLP1	BC097516	1.176	1	PM
ribosomal protein S4	TDITYPAGFMDVISIEK	RPS4	BC106700	1.175	1	PM
adenosine kinase a	AGCTLPEKPDFI	ADKA	BC075155	1.175	1	RN
nuclear phosphoprotein	DNDSCPTSLLDIFSDWVK	xNopp180	NM_001088086	1.174	1	UNK
protein phosphatase 2 regulatory subunit A	NDLIPLFTNLASDEQDSVR	PPP2R1A	BC078080	1.174	1	CC,ST
ribosomal protein S16	LLEPVLLLGK	RPS16	NM_001016347	1.173	1	PM
similar to ribophorin II	LLVTNVLSQPLTEAK	RPN2	BC046727	1.173	1	PM
similar to histone H2b	NSFVNDIFER	H2B	BC077399	1.172	1	RN

TABLE 3 (continued)

PROTEINS EXPRESSED AT ELEVATED LEVELS IN 0dPA RELATIVE TO 3dPA

Protein Identification by Peptide(s)	Peptide	UniGene	Accession	FC	Category	Biological Process
ribosomal protein L10	VDEFPLCGHMVSDEYEQLSSEALEAAR	RPL10	BC044716	1.169	1	PM
heteronuclear ribonucleoprotein H2	ATETDIYTFFSPLNPVR	HNRPH2	BC074690	1.168	1	RN
similar to myelin expression factor 2	NLPFDLTWQK	MYEF2	NM_001016035	1.166	1	RN
ribosomal protein S8	LDAGNFSWGSECCTR	RPS8	BC054266	1.162	1	PM
splicing factor U2AF large chain	LFIGGLPNYLNDDQVK	U2AF2	BC044032	1.162	1	RN
DNA replication licensing factor 5	LQPFATETDVEEALR	MCM5	BC059310	1.162	1	RN
ribosomal protein SA	AIVAIENPADVCVISSR	RPSA	BC061298	1.161	1	CC,ST
glutathione-S-transferase, mu1	FEALPAIDAYMK	GSTM1	BC053774	1.161	1	M,EP
histone H3a	GLFEDTNLCAIHAK	H3A	NM_001097650	1.161	1	RN
glutathione-S-transferase, mu 2	LGLDFPNLPYLVDGDVK	GSTM2	BC053774	1.160	1	M,EP
acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	CPNITYLNLSGNK	ANP32E	BC094475	1.158	1	UNK
nucleolin	VLVVNNLSYSATEDSLR	NCL	X72957	1.158	1	RN
dynein light chain, LC8-type 2	NADMSEEMQQDAVECATQALEK	DYNLL2	BC068877	1.158	1	CG,M
U2 small nuclear ribonucleoprotein auxiliary factor 2	LGGLTQAPGNPVLAVQINQDK	U2AF2	BC067966	1.155	1	RN
chaperonin-containing TCP1 subunit 2	VQDDEVGDGTTSVTVLAAELLR	CCT2	NM_001086919	1.151	1	PM
translation elongation factor-1 gamma	WFVTCVNQPEFR	EEF1G	NM_001088012	1.144	1	PM
poly(rC) binding protein 2, hnRNP-E2 protein	IITLAGPTNAIFK	PCBP2	BC084195	1.144	1	RN
ribosomal protein L5	NGVTADQVEDLYK	RPL5	BC041227	1.143	1	PM
DEAD-box polypeptide 5	MLDMGFEPQIR	DDX5	BC063223	1.143	1	RN
ribosomal protein L4	IEEIPEVPLVVEDK	RPL4	BC106315	1.142	1	PM
similar to ribosomal protein L24	AITGASLAEIMAK	RPL24	Y17113	1.140	1	PM
similar to heterogeneous nuclear ribonucleoprotein D-like	QYFGGFGEIENIELPIDTK	HNRPD	BC045124	1.140	1	RN
similar to heterogeneous nuclear ribonucleoprotein G	LFIGGLNTETNEK	HNRPG	BC070649	1.139	1	RN
chaperonin-containing TCP1 subunit 8	FAEAFESIPR	CCT8	NM_001087244	1.137	1	PM
ribosomal protein S12	LGEWVGLCK	RPS12	NM_001087125	1.137	1	PM
annexin A6	VILGLMMTPAQFDAK	ANXA6	BC073422	1.136	1	CC,ST
ribosomal protein L23a	LAPDYDALDVANK	RPL23A	BC087796	1.135	1	PM
malate dehydrogenase 1, NAD (soluble)	IVEGLCINDFSR	MDH1	BC075396	1.133	1	M,EP
ribosomal protein L11	VLEQLTGQTPVFSK	RPL11	BC135522	1.133	1	PM
H2A histone family, member Y2	EVASSSTSDDGPGDAFTILSSK	H2AFY2	BC056065	1.133	1	RN
heterogeneous nuclear ribonucleoprotein U scaffold attachement factor A	YNILGTNTIMEK	HNRPU	NM_001097364	1.131	1	RN
similar to prohibitin 2 isoform 1	VPWFQYPIIYDIR	PHB2	NM_001092833	1.125	1	RN
keratin 16	IGSLEEQLQQIR	KRT16	NM_001086376	1.123	1	CG,M
retinoblastoma-A associated protein, RbAp48	YMPQNPCIIATK	RBBP4	AF073787	1.121	1	RN
54kDa nuclear RNA-binding protein	FGQAPNIEGLGGANPPAFPR	P54NRB	BC045128	1.119	1	RN
translation elongation factor-1 alpha	SGDAAIVDMIPGKPMCVESFSDYPPLG	EEF1A	BC043843	1.119	1	PM
histone H2A	EGASSSTSDDGPGDAFTILSSK	H2A	BC076893	1.118	1	RN
ubiquitin-activating enzyme E1	VYDDDFFEALDGVANALDNIDAR	UBE1	AB040073	1.118	1	PM
cleavage and polyadenylation specific factor 6, 68kDa	AVSDASAGDYGSAIETLVTAISLIK	CPSF6	BC077388	1.116	1	RN
DNA replication licensing factor 4	SLNPEDIDQLITISGMVIR	MCM4	BC074670	1.116	1	RN
heterogeneous nuclear ribonucleoprotein D-like	VFVGGLSPETTEEQIK	HNRPD	BC045124	1.115	1	RN
thioredoxin domain containing 5	DLETLQNYVLR	TXNDC5	BC045245	1.115	1	PM
RNA and export factor binding protein 2	MDMSLDDIIK	REFBP2	BC135842	1.114	1	UNK
arginine methyltransferase-1b	DVAIKEPLVDVVDPK	PRMT1	BC072069	1.114	1	M,EP
RNA-binding protein Vera	ITISPLQDLTLYNPER	VERA-A	AF055923	1.110	1	UNK
chaperonin containing TCP1 subunit 6A (zeta 1)	VLAQNSGYDPQETLVK	CCT6	BC084219	1.110	1	PM
chaperonin-containing TCP1 subunit 7	LPIGDVATQYFADR	CCT7	NM_001093568	1.109	1	PM
translation initiation factor-3 subunit 10	DIDIENLEELDPDFIMAK	EIF3S10	BC043785	1.108	1	PM
retinoblastoma binding protein 7, RbBP7	TPSADVLVFDYTK	RBBP7	BC064219	1.108	1	RN
telomerase binding protein, p23	YLNEVELFQSIDPNASK	TEBP	BC084900	1.104	1	UNK
ras-GTPase-activating protein SH3-domain-binding protein	LPNFGFVVFDDAEPVQK	G3BP	BC045051	1.102	1	CC,ST
DNA unwinding factor 87, DUF87	SENPGISITDLSK	SSRP1	BC082613	1.101	1	RN
ribosomal protein L23	LPVGAVINCADNTGGK	RPL23	BC073541	1.100	1	PM
pre-mRNA processing factor-8	LANQLLTDLVDDNYFYLFDLK	PRP8	NM_001086784	1.100	1	RN
20S proteasome alpha1 subunit	YGYEIPVDMLCK	LOC446974	BC084423	1.096	1	UNK
translation elongation factor-2	IWCFGPDGSGPNILTDVTK	EEF2	NM_001087187	1.095	1	PM
pre-mRNA processing factor-19 homolog	SASVPGILALDLCPTDTNK	PRPF19	BQ732328	1.094	1	RN
septin A	STLINSLFLTDLYPER	SEPTA	AF212298	1.094	1	UNK

TABLE 3 (continued)

PROTEINS EXPRESSED AT ELEVATED LEVELS IN 0dPA RELATIVE TO 3dPA

Protein Identification by Peptide(s)	Peptide	UniGene	Accession	FC	Category	Biological Process
ribosomal protein S1	DEIVPTTPISEQK	RPS1	BC130166	1.092	1	UNK
pleckstrin homology domain containing, family C member 1	TSTILGDITSIPELTDYLK	PLEKHC1	BC077819	1.223	2	UNK
26S proteosome non-ATPase regulatory subunit 14	LGGGMPGLGQGPPTDAPAVDTAEQVYISSLALLK	PSMD14	BC045094	1.223	2	PM
valyl-tRNA synthetase like	LLSPFMPFLTEELYQR	VARS2L?VARSL	BC084762	1.222	2	M,EP
DNA replication licensing factor 4	SLNPEDIDQLITISGMVIR	MCM4	BC074670	1.222	2	RN
dynein light chain, DLC8a	DIAAFIK	DYNLL2	BC088794	1.214	2	CG,M
FHA domain-interacting nucleolar phosphoprotein-like	SLIEPQLQEYFNQFGTVTR	MKI67IPL	BC122487	1.201	2	UNK
SET translocation	VEVTEFEDIK	SET	CK740651	1.197	2	RN
keratin 18	LEMLGGTAMALETELVQ	KRT18	Y00230	1.197	2	CG,M
transcription factor IID subunit	SIAGLAELGPDVV	TAF6	BC068776	1.190	2	RN
spermatid perinuclear RNA binding protein	GWPLELICEK	STRBP	BC078118	1.185	2	RN
ubiquitin-conjugating enzyme E2 variant 1	NPDGTMNLMNWECAIPGK	UBE2V1	BC045066	1.184	2	CD
nuclear receptor coactivator 5	GVAPPGIQAVLGLLADNR	NCOA5	BC044688	1.181	2	RN
poly(rC) binding protein 3	LVVPASQCGSLIGK	PCBP3	CR855568	1.180	2	RN
Williams-Beuren syndrome chromosome region 1	ELPTEPPFTAYVGNLPFNTVQGDIDNIFK	WBSCR1	BC078074	1.179	2	UNK
similar to heterogeneous ribonuclear particle protein	SYGGDGFGNDGGFGGSPPYSGGNR	HNRPA1	BC045260	1.178	2	RN
replication protein A3	NATVELNEPLEEEISGIIEVIGK	RPA3	BC077497	1.178	2	RN
golgi phosphoprotein 3	ETPTPETVQSWIELLSGETWNPLK	GOLPH3	CR760594	1.176	2	T
creatine kinase, brain	GGDDLDPNYVLSSR	CKB	BC042282	1.176	2	M,EP
similar to 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMGCoA reductase	FLLLIDLSR	HMGCR	BC074197	1.174	2	M,EP
DNA unwinding factor 140kDa subunit	TLMVDPTQEMQENYNFLLQLQEELLK	SUPT16H	NM_001090697	1.168	2	RN
RAN-binding protein 7	MDPNILIEALRGTM	IPO7	BC046568	1.166	2	T
similar to PR domain containing 2, with ZNF domain	NIPQTFTTAIR	PRDM2	BC100183	1.166	2	RN
methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1	FLYDVQLPIADK	MTHFD1	BC080885	1.166	2	M,EP
5'-3'-exoribonuclease-2	FAWQGVALLPFVDER	XRN2	CR848514	1.166	2	RN
wnt inhibitory factor-1	VNVIVMNSEGNVILQ	WIF1	AF122924	1.163	2	CC,ST
146kDa nuclear protein	EFGAGPLFNQILPLLMSPTLEDQER	SF3B1	BC097718	1.162	2	RN
DNA-dependent RNA polymerase subunit	PSEGVTGDLIINNNV	MGC80013	BC070564	1.162	2	UNK
similar to transmembrane protein 6	LFLIMLVLK	TMED6	BC084087	1.162	2	T
SAPS domain family, member 2	LLKNMFDGE	SAPS2	BC073386	1.162	2	UNK
armadillo repeat containing protein 6	MIVLENGGLK	ARMC6	BC076906	1.161	2	SS,ST
histone H1A variant	AAAAAGAAK	H1A	NM_001095702	1.160	2	RN
ubiquitin-activating enzyme E1	VYDDDFFEALDGVANALDNIDAR	UBE1	AB040073	1.159	2	PM
RAN-binding protein 1	FASENDPPEWK	RANBP1	BC054182	1.159	2	RN,ST
small nuclear ribonucleoprotein polypeptide D3	FLILPDMLK	SNRPD3	BC056127	1.158	2	RN
5-aminoimidazole-4-carboxyamide ribonucleotide formyltransferase/IMP cyclohydrolase	DVSEITGFPEMLGGR	ATIC	BC106381	1.157	2	M,EP
nuclear cap-binding protein subunit 1-B	STSSLESNLEGLAGVLEADLPNYK	NCBP1B	BC072867	1.155	2	UNK
glypican-4	LEGPFNIETVMDPIDVK	GPC4	BC090231	1.154	2	CC,ST
4-hydroxyphenylpyruvate dioxygenase	LDYNGIFLPGYEPPLFQDPLLPK	HPD	BC084120	1.147	2	M,EP
similar to ribosomal protein S25	LNNLVLFDK	RPS25	NM_001005084	1.146	2	PM
structure specific recognition proptein 1	FGGQLLSFDIGDQPAFELPLSNVSQCTTGK	SSRP1	BC098960	1.144	2	RN
ribosomal protein L26	FSPFVTSDR	RPL26	NM_001005104	1.142	2	PM
tripeptidyl peptidase II	EMLNYSVNIYDEGNLLSVVTSGGAHG	TPP2	BC071158	1.137	2	PM
mitotic phosphoprotein 22	TNENLEDVEASK	MP22A	AF419149	1.134	2	UNK
ribonuclease H2 large subunit	SSTPSVLSFFSAPK	RNASEH2A	BC061614	1.134	2	RN
nucleophosmin/nucleoplasmin, 3	LSCQPMVNMGSFEIEAPVTFR	NPM3	BC043908	1.131	2	PM
translation initiation factor-2 subunit 3	IVLTNPVCTEVGEK	EIF2S3	BC043966	1.128	2	PM
ribosomal protein S28	EGDVLTLLESER	RPS28	BC080501	1.125	2	PM
similar to cytochrome c oxidase subunit IV isoform 2	AFPLPDIPFQTELSSQQVTLK	COX4I2	BC075214	1.109	2	M,EP
translation initiation factor-3 subunit 10	LSSLLPFVDAFLLER	EIF3S10	BC043785	1.108	2	PM
translation initiation factor-3 subunit 6-interacting protein	GDAQIYEELFNYACPK	EIF3S6IP	NM_001086876	1.103	2	PM
similar to basic transcription factor 3-like 4	QITEMLPGILSQLGADSLTSLR	BTF3L4	BC087817	1.099	2	UNK
U6 snRNA-associated protein	YVQLPADEVDTQLLQDAAR	LSM2	BC090606	1.095	2	RN
NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	GDLPPLTLENIAER	NDUFA1	BC106594	1.095	2	M,EP
voltage-dependent anion channel-2	LTFDTTFSPNTGK	VDAC2	NM_001016193	1.093	2	T
S-phase kinase associated protein-1A isoform b	TDDIPVWDQEFLK	SKP1A	BJ092310	1.092	2	PM

TABLE 3 (continued)

PROTEINS EXPRESSED AT ELEVATED LEVELS IN 0dPA RELATIVE TO 3dPA

Protein Identification by Peptide(s)	Peptide	UniGene	Accession	FC	Category	Biological Process
Novel, Hypothetical,or Unknown Proteins						
	DLSPFGELYEMLK	LOC734164	BC100168	1.366	2	
	ILPNLPGPK	MGC82736	BC073514	1.337	2	
	KAPQQQPAASTSAQTKR	LOC733957	NP_001039134	1.244	2	
	STSEDATSESKR	MGC:85118	BC091716	1.215	2	
	CLPVNPCPANPR	LOC495265	BC084326	1.205	2	
	SVAEPENPDHVAEPVTSEGLQQSQTETGK	MGC86492	BC081276	1.199	1	
	YNDSSSQQLR	MGC83738	BC070741	1.175	2	
	DLSPFGELYEMLK	IMAGE:4960177	BC121192	1.139	1	
	LSGLTEVTQLLQEPLNPEQK	MGC:115513	BC097801	1.118	2	
	DLDDVVNSGLAK	LOC398653	BC053763	1.088	2	

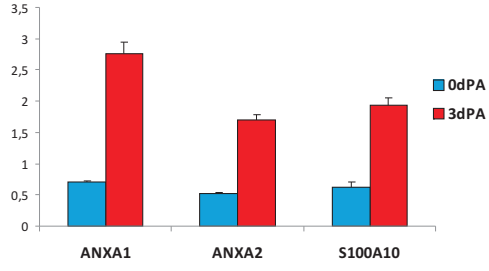
Protein names are those determined from the peptide of highest confidence with the peptide sequence shown. The proteins are sorted by FC with category 1 peptides listed first followed by category 2. Only proteins with FC above that of the internal standard are listed. Accession numbers are those sequences that identified the peptide of highest confidence. Function refers to "Biological Process" as defined by the Human Protein Reference Database (HPRD). Protein names (or UniGene ID for unknowns) listed in bold type are those whose encoding gene was also found expressed at higher levels in stage 53 1dPA versus 3dPA (unpublished). Abbreviations: CG,M = cell growth and/or maintenance; CC,ST = cell communication, signal transduction; T = transport; PM = protein metabolism; M,EP = metabolism, energy pathways; RN = regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism; CP = cell proliferation, anti-apoptosis, cell growth and/or maintenance; CD = cell differentiation; DNAR = DNA repair; RL = RNA localization; UNK = unknown; FC = fold change.

shown in Table 5 the fold increase from 0dPA to 3dPA is similar for both the mRNA and the protein as determined by LC/MS proteomics and qPCR. All three of these genes were also detected as differentially expressed in our gene array screen (Grow *et al.*, 2006). Microarray analysis (unpublished) showed a 2.8-fold increase in *ANXA1*, a 2-fold increase in *ANXA2*, and a 4-fold increase in *S100A10* in regeneration-competent 3dPA versus 1dPA blastemas. Although a comparison of gene expression differences between 1dPA and 3dPA is clearly not identical to the 0dPA and 3dPA proteomics comparison, we can infer that many proteins whose levels are increased from 0dPA to 3dPA might also be reflected in an increase in gene expression between 1dPA and 3dPA.

We showed previously by RT-PCR and qPCR analysis that several 3dPA immune-related genes (*SOCS3*, *MyD88*, *gp96*, and *FGL2*) were also greatly up-regulated relative to their levels in intact limbs (Grow *et al.*, 2006). In comparing the pattern of expression of these immune genes in the regeneration-competent limb (stage 53) to that in the non-competent limb (stage 57), we found that the amputation-induced expression was maintained to a much greater extent by 5dPA at stage 57. We therefore asked if the expression of *ANXA1*, *ANXA2* and *S100A10* showed a similar pattern. The expression of *ANXA1*, *ANXA2* and *S100A10* was assessed in stage 53 hindlimbs at 0, 1, 3, and 5 days after amputation and compared to expression in similar tissue from hindlimbs at stage 57. All three genes are up-regulated from 3- to 5-fold within one day of amputation in limbs at both stages (Figure 3). In stage 53 limb stumps their expression is maximal 3 days after amputation (5- to 9-fold) but by 5 days had returned to levels similar to those present at 0 day, possibly reflecting resolution of inflammation as competent blastemas form. In stage 57 hindlimbs undergoing hypomorphic regeneration, the induced expression of these three genes peaks by 1 day after amputation (3- to 5-fold increases), but also returns to near baseline levels by day 5. Thus, the expression of annexins and S100A10 exhibits a pattern distinct from that of other immune-related genes that we have analyzed following hindlimb amputation.

Proteins expressed in stem cells

Since cells of the distal amputated limb stump undergo dedifferentiation and proliferate to form the regeneration blastema and the relationship of blastema cells to stem cells is not clear, we also



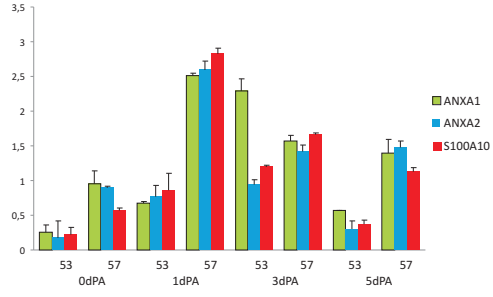


Fig. 2 (Left). qPCR analysis of gene expression. *Graphical representation of the qPCR determined level of expression of ANXA1, ANXA2 and S100A10 in 0dPA and 3dPA stage 53 limb tissues normalized to the expression of ODC. Error bars represent the standard deviation from triplicate assays.*

Fig. 3 (Right). qPCR analysis of gene expression. *Graphical representation of the qPCR determined level of ANXA1, ANXA2 and S100A10 expression, normalized to the expression of ODC, following limb amputation at regeneration-competent and incompetent stages. Expression levels of ANXA1 and ANXA2 and the ANXA2-binding partner S100A10 were assayed by qPCR. 0dPA, 1dPA, 3dPA and 5dPA refer to days post-amputation. 53 and 57 refer to* Xenopus *stages 53 and 57 respectively. Error bars represent the standard deviation from triplicate assays.*

wished to determine whether any of the abundant proteins significantly up-regulated 3dPA have been identified consistently in stem cells. Proteins listed in Table 2 were compared to those identified in mammalian stem cells primarily by proteomic analyses and recently reviewed by Baharvand *et al*. (2007) and others. As shown in Table 6 nearly thirty of the abundant proteins of various functional classes up-regulated during blastema formation are also expressed in one or more of these stem cells. Eight of these proteins have also previously been shown by gene expression microarray data to undergo increased synthesis after amputation of stage 53 limbs (Grow *et al*., 2006).

Discussion

LC/MS proteomic analysis of unfractionated tissue from stage 53 *Xenopus* hindlimbs at the time of and 3 days after amputation identified and quantified a total of 1517 mostly abundant peptides. Of these, 365 proteins were identified by multiple peptides each with a high level of confidence and found differentially expressed when comparing the two time points. The peptide sequences identified a total of 145 proteins which were found to be higher in 3dPA tissue compared to 0dPA (Table 2). These include proteins involved in many biological processes, with factors related to aspects of innate immunity or inflammation well represented.

One measure of the validity of the proteomic approach we used is the fact that we identified several proteins significantly more concentrated in 3dPA blastemas which have been previously shown to be up-regulated during limb regeneration by other investigators using other species and other analytical methods (Geraudie and Ferretti, 1998). These include integrin αV (ITGAV), matrix metalloproteinase 9 (MMP9), collagen type I (COL1A1), fibronectin (FN1), vimentin (VIM) and specific keratins (KRTs). Approximately half of those listed in Table 2 were also identified among genes with significantly up-regulated expression in limb blastemas, as detected by *Xenopus laevis* microarrays (Grow *et al*., 2006) or other methods with *Ambystoma* limbs (Putta *et al*., 2004) or teleost fins (Katogi *et al*., 2004).

A previous proteomic comparison of intact and regenerating adult newt limbs analyzed expression of specific proteins by quantitative 2-D gel electrophoresis and autoradiography (Tsonis *et al*., 1992). One protein up-regulated approximately 10-fold in the early blastema was identified by partial sequencing as a homolog of *Xenopus* keratin B2, which we also found induced in this study (Table 2). Database comparison of the partial sequence reported by Tsonis *et al*. (1992) reveals further homology to newt keratin 17 (KRT17), the keratinocyte-specific antigen recognized

TABLE 4

PROTEINS FROM TABLE 2 HAVING ROLES IN IMMUNE FUNCTION AND/OR MODULATION

Protein Name	Reference
annexin 1	(Parente and Solito, 2004)
annexin 2, annexin II type 1	(Abbas *et al*., 2005)
annexin 5	(Munoz *et al*., 2007)
B fibrinopeptide	(Mosesson *et al*., 2001)
calpactin I (annexin II) light chain p11 subunit	(Laumonnier *et al*., 2006)
complement component 1 q subcomponent binding protein	(Abbas *et al*., 2005)
elastase 2	(Horwitz *et al*., 2007)
fibrinogen alpha	(Ugarova and Yakubenko, 2001)
fibrinogen gamma	(Ugarova and Yakubenko, 2001)
gelatinase B, matrixmetalloproteinase 9	(Abbas *et al*., 2005)
granulin	(Ong *et al*., 2006)
integrin alpha V subunit	(Schoeler *et al*., 2003)
interferon regulatory factor 2 binding protein 2	(Childs and Goodbourn, 2003)
lymphocyte cytosolic protein 1	(Abbas *et al*., 2005)
manganese superoxide dismutase	(Abbas *et al*., 2005)
moesin	(Abbas *et al*., 2005)
myeloperoxidase, peroxidase 2	(Rutgers *et al*., 2003)
phosphatidylserine decarboxylase	(Abbas *et al*., 2005)
prohibitin	(Theiss *et al*., 2007)
putative cathepsin L	(Abbas *et al*., 2005)
slingshot-related protein	(Abbas *et al*., 2005)
transaldolase 1	(Abbas *et al*., 2005)
transferrin, lactotransferrin	(Weinberg, 2007)
vimentin (VIM1, VIM4)	(Benes *et al*., 2006)

Protein names listed in bold type are those whose encoding gene was also found expressed at higher levels in stage 53 3dPA versus 1dPA (unpublished).

TABLE 5

COMPARISON OF THE FC VALUES FOR ANXA1 AND ANXA2 AND THE ANXA2-BINDING PARTNER, S100A10 DETERMINED BY PROTEOMICS AND QPCR

	ANXA1	ANXA2	S100A10
qPCR	3.93	3.26	3.10
Proteomics	1.76	1.21	1.26

TABLE 6

PROTEINS FROM TABLE 2 THAT ARE ALSO EXPRESSED AT ELEVATED LEVELS IN VARIOUS MAMMALIAN STEM CELL TYPES

Protein Name	SC Types	References
actin beta	MSC, NSC	(Baharvand *et al*., 2007)
actin gamma	MSC, NSC	(Baharvand *et al*., 2007)
aldolase A	ESC, MSC	(Baharvand *et al*., 2007)
annexin 1	MSC, NSC	(Baharvand *et al*., 2007)
annexin 2, annexin II type 1	MSC, NSC	(Baharvand *et al*., 2007)
annexin 5	MSC, NSC	(Baharvand *et al*., 2007)
annexin 7	MSC	(Kim *et al*., 2006)
ATP-dependent chromatin remodeling DEXH-box protein	NSC	(Matsumoto *et al*., 2006)
ferritin	ADSC	(Zvonic *et al*., 2007)
glutathione-S-transferase	ADSC	(Zvonic *et al*., 2007)
glyceraldehyde-3-phosphate dehydrogenase type B	MSC, NSC	(Baharvand *et al*., 2007)
keratin	ADSC	(Zvonic *et al*., 2007)
manganese superoxide dismutase	ADSC	(Zvonic *et al*., 2007)
NM23/nucleoside diphosphate kinase	ADSC	(Zvonic *et al*., 2007)
prohibitin	ESC, NSC	(Baharvand *et al*., 2007)
prosaposin	ADSC	(Zvonic *et al*., 2007)
putative cathepsin L	ADSC	(Zvonic *et al*., 2007)
serum albumin B precursor	MSC, NSC	(Baharvand *et al*., 2007)
SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily d, member 1	ESC	(van Hoof *et al*., 2007)
transaldolase 1	ADSC	(Zvonic *et al*., 2007)
tropomyosin	MSC, NSC	(Baharvand *et al*., 2007)
vimentin (VIM1, VIM4)	MSC, NSC	(Baharvand *et al*., 2007)

Protein names listed in bold type are those whose encoding gene was also found expressed at higher levels in stage 53 3dPA versus 1dPA (unpublished). MSC = mesenchymal stem cells; NSC = neural stem cells; ESC = embryonic stem cells; ADSC = adipose tissue stem cells.

by monoclonal antibody WE6. Estrada *et al.* (1993) localized WE6 expression in glands of newt skin and other mucosae, with weak staining in endothelium and smooth muscle. In limb blastemas KRT17 was strongly expressed in the wound epidermis but completely absent in underlying mesenchymal cells, and this same pattern of WE6 expression was also seen in amputated non-regenerating forelimbs of post-metamorphic *Xenopus* and *Rana* (Estrada *et al.*, 1993).

In mammals KRT17 is also expressed in epidermal appendages (hair follicles, nail beds, sebaceous glands, and sweat glands, including myoepithelial cells) but in interfollicular keratinocytes only after acute injury or inflammation (McGowan and Coulombe, 1998). Kim *et al.* (2006) have shown that KRT17 regulates protein synthesis and cell growth during keratinocyte migration and epithelial wound closure, an effect mediated by mTOR kinase with binding of KRT17 to the adaptor protein 14-3-3σ. This work suggests that KRT17 and other intermediate filament proteins that provide modulated structural support during cell migration also influence cell growth and death through dynamic interactions with non-structural proteins (Coulombe and Wong, 2004).

Another keratin up-regulated in our study is KRT8, which has been previously shown by Ferretti to be transiently expressed in dedifferentiating mesenchymal cells 3-5 days after amputation in adult newt limbs (Corcoran and Ferretti, 1997). Although KRT8 is well-known to dimerize with KRT18 as part of the cytoskeleton in simple epithelial cells, it has also been found to associate with ANXA1, suggesting a role in modulation of the inflammatory response (Croxtall *et al.*, 1998; Rual *et al.*, 2005).

This proteomics study found increased levels of numerous inflammatory factors following amputation, which is consistent with the gene expression profiles of regenerating amphibian or fish appendages (Grow *et al.*, 2006; Lien *et al.*, 2006; Schebesta *et al.*, 2006). These include annexins, anti-oxidant proteins, complement components, galectins, granulin, cathepsins, vimentin, and factors that regulate transforming growth factor-β (TGF-β) signaling. Immunohistochemistry indicates that vimentin is localized primarily in scattered cells of stage 53 limbs and blastemas, particularly in Langerhans-like cells of the epidermis (Mescher *et al.*, 2007). Up-regulation of many such inflammation-related genes is greater in limbs of prometamorphic *Xenopus* at regeneration-incomplete stages than in limbs at stage 53, which regenerate well (Grow *et al.*, 2006). One interpretation of these results is that the developmental state of the anuran immune system and the differentiation status and number of cells mediating the inflammatory response to trauma may determine the nature of the regenerative response to amputation (Harty *et al.*, 2003; Mescher and Neff, 2005; Mescher and Neff, 2006), a hypothesis consistent with current work on scar formation after injury to developing mammalian skin (Martin and Leibovich, 2005).

Among the proteins showing a substantial increase three days after amputation at stage 53 are members of the annexin family, particularly ANXA1 and ANXA2, all of which were identified with multiple sequences. Annexins bind phospholipids in a Ca^{2+}-dependent manner and help regulate a wide variety of cellular activities (Gerke and Moss, 2002). ANXA1, whose expression is up-regulated by glucocorticoids, is one of several anti-inflammatory mediators that operate locally to ensure the transient profile of the inflammatory reaction, i.e. to prevent chronic inflammation with its potential for tissue damage (Perretti and Flower, 2004). ANXA1 and ANXA2 lack signal peptides but can be exported to the cell surface or ECM in a dimeric complex with S100A10 (Svenningsson and Greengard, 2007) another protein found to be up-regulated at 3dPA (Table 2). Complexed with S100A10 on the cell surface, ANXA2 binds plasminogen and promotes other key effects during inflammation, including the localized generation of plasmin important for focused degradation of the ECM and cellular invasiveness (Kwon *et al.*, 2005a); stimulates the release of the angiogenesis inhibitor angiostatin as plasminogen undergoes autoproteolytic cleavage (Kwon *et al.*, 2005b); and triggers the plasmin-induced release of tissue factor (TF), IL-1, TNF-α, monocyte chemoattractant protein-1, and other factors from monocytes (Laumonnier *et al.*, 2006).

The important roles of ANXA1 and ANXA2 as highly localized anti-inflammatory factors make these proteins prime candidates not only in regulating local activities in the amputated limb but also in determining whether inflammation is short-lived or prolonged. ANXA2 expression is up-regulated during both tail (Tazaki *et al.*, 2005) and limb (Grow *et al.*, 2006) regeneration in larval *Xenopus*. The activity of the ANXA2-S100A10 heterotetramer in stimulating plasmin-induced expression of TF in activated monocytes (Laumonnier *et al.*, 2006) may be important for the localized production of TF and thrombin required for initiating regeneration in both newt limbs (Morais da Silva *et al.*, 2002) and lens (Godwin and Brockes, 2006; Imokawa and Brockes, 2003). Such studies add considerable interest to the ANXA2-S100A10 heterotetramer as an inflammatory component to be examined during regeneration.

We show here that expression of ANXA1, ANXA2, and S100A10 is up-regulated within one day after amputation in limbs of both stage 53 and stage 57 larvae (Figure 3). Expression of all three genes remains elevated 5 days post-amputation in limbs of stage 57 larvae, but declines during this period in limbs of stage 53 larvae, an expression pattern shared with several other inflammation-related genes (Grow *et al.*, 2006). These results are consistent with the view that inflammation is prolonged in the "pseudoblastemas" of regeneration-incompetent limbs, but is resolved more quickly in the limb stumps of stage 53 larvae which regenerate almost completely. Taken together with evidence from mammalian studies (Perretti and Flower, 2004), it is highly suggestive of a role for ANXA1 in determining the course of inflammation and its resolution.

Microarray studies of mammalian wound healing and the use of transgenic or knockout models for specific leukocytes or cytokines have made increasingly clear that the inflammatory phase of repair determines the extent of scar formation or the quality of tissue regeneration (Harty *et al.*, 2003; Cooper *et al.*, 2005; Martin and Leibovich, 2005; Mescher and Neff, 2005; Eming *et al.*, 2007). Such studies also reveal that activation of pro-inflammatory genes and cytokines is generally accompanied by activation of anti-inflammatory genes and expression of mediators which down-regulate aspects of the inflammatory response (Babbin and Gewirtz, 2005; Martin and Leibovich, 2005). The balance of activities between pro- and anti-inflammatory factors characterizes the normal sequence of inflammatory events, including recruitment of leukocytes, killing and removal of potential pathogenic invaders, and removal of dead tissue, followed by an

active process of resolution that ends these activities (Ariel and Serhan, 2007). Circumstances which produce excessive or prolonged pro-inflammatory activity can lead to chronic wounds or excessive scarring. In addition to fetal skin, experimental manipulations that reduce the number of inflammatory cells, or signaling from certain mediators such as TGF-β after wounding, lead to faster repair, reduced scarring, and possible restoration of normal tissue architecture (Harty *et al*., 2003; Martin and Leibovich, 2005).

Finally, since cells of the distal amputated limb stump undergo dedifferentiation and proliferate to form the regeneration blastema and, the relationship of blastema cells to stem cells is not clear, we also wished to determine whether any of the abundant proteins significantly up-regulated in 3dPA have been identified consistently in stem cells. Proteins listed in Table 2 were compared to those identified in mammalian stem cells primarily by proteomic analyses and recently reviewed by Baharvand *et al*. (2007) and others. As shown in Table 6 nearly thirty of the abundant proteins of various functional classes up-regulated during blastema formation are also expressed in a variety of cultured mammalian stem cells. Expression of these blastema proteins was most commonly shared with mesenchymal and adipose-derived stem cells. Eight of these proteins up-regulated in both blastema and stem cells have also been shown to have increased gene expression in limbs following amputation (Grow *et al*., 2006). Table 6 only includes proteins from categories 1 and 2 that are significantly up-regulated 3 days after amputation; proteins in category 3 (identified from multiple peptides with a confidence of 75-89%) include several others with expression shared by stem cells, including Tpt1 (translationally controlled tumor protein-1, TCTP), which was recently found to be an activator of the *oct4* and *nanog* pluripotency transcription factors during reprogramming of transplanted somatic nuclei in *Xenopus* eggs (Koziel *et al*., 2007). Further analysis of proteins expressed in both the blastema and multiple stem cells should yield insights into the nature of cellular dedifferentiation in the limb stump and the possible involvement of stem cells in limb regeneration.

This proteomic study, together with the screens of gene expression during early regeneration, reveals that both pro-inflammatory and anti-inflammatory activities are up-regulated in amputated amphibian limb stumps. This raises the likelihood that ideas emerging from recent work on mammalian wound healing regarding the control of scarring and tissue regeneration will prove applicable to limb regeneration and will yield greater understanding of the ontogenic decline of regeneration in anurans. Together with comparisons of proteomic and gene array analyses of stem cells, further work on the role of inflammation during the early phase of regeneration will yield additional insights into the molecular bases of dedifferentiation and other aspects of epimorphic regeneration.

Materials and Methods

Limb amputation and blastema collection

Larval *Xenopus laevis* were raised in the laboratory or obtained commercially (NASCO, Ft. Atkinson, WI.) and hindlimbs were staged according to Nieuwkoop and Faber (1967). All surgical procedures were performed according to procedures approved by the Indiana University Animal Care and Use Committee following anesthesia in 0.005% benzocaine. Hindlimbs at stage 53 (when regeneration is essentially complete) were amputated bilaterally at the mid-zeugopodia. Immediately (0dPA) and 3 days (3dPA) after amputation, tissues were collected 1mm proximal to the original plane of amputation and pooled for proteomic analysis.

Tissue preparation, LC/MS/MS and data analysis

All processing of tissues, mass spectrometry and statistical analysis of data was carried out under contract with Monarch Life Sciences, LLC (Indianapolis, IN.), formerly the Indiana Centers for Applied Protein Science (INCAPS), a fee-for-service contract research company formed through Indiana University, Eli Lilly & Co, and other organizations. A total of 10 pools of tissue each from 0dPA and 3dPA limbs stumps was collected and processed for protein digestion. Each pool of tissue consisted of 120 hindlimb or blastema pieces. Tissues were homogenized and the resultant lysates were reduced and alkylated by triethylphosphine and iodoethanol and then digested with trypsin as described (Higgs *et al*., 2005 and 2007). Peptide concentration in each pool was determined by the Bradford protein assay. All procedures for quantification of proteins, assurance of quality of the results and statistical analysis were carried out according to the detailed steps outlined by Fitzpatrick *et al*. (2007) and Monarch Life Sciences, LLC. Details of the Methods used in this publication are included as supplementary materials.

Reverse-transcription and quantitative PCR (qPCR)

Analysis of the expression of several annexin genes and S100A10 was carried out using quantitative RT-PCR essentially as described by King *et al*. (1998). Total RNA samples were extracted using the RNAqueous Micro system (Ambion, USA). Reverse transcription reactions were carried out using 1μg of total RNA purified from indicated sources. Each qPCR was carried out using the equivalent of 2.8 ng of input RNA. As a control for RNA loading into the RT reaction, expression of *Xenopus laevis* ornithine decarboxylase (ODC) was assayed (King *et al*., 1998). The primer sequences are: ANXA1 (accession BC053786) upstream: 5'-cagatctgcattcagtccttgatc-3', downstream: 5'-gcttgcctacgctcttttggtaag-3'; ANXA2 (accession M60768) upstream: 5'-ggaaatcaatcatgtgcaaggcag-3' downstream: 5'-tttccagattgccagaaagcgctc-3'; S100A10 (accession M538593) upstream: 5'-tgcaacgagtattacgtcaaacac-3', downstream: 5'-atggagggtaaatctggaatagag-3'.

Quantitative PCR was performed utilizing the Mx3000P QPCR System (Stratagene, USA.). Fluorescence detection chemistry involved utilization of SYBR green dye master mix (Bio Rad, USA.) and was carried out as described (Grow *et al*., 2006). Each RT reaction was equalized for RNA input by assessing the level of expression of the relatively invariant housekeeping gene ornithine decarboxylase (ODC) and quantitative expression of each gene of interest was then normalized to the level of ODC.

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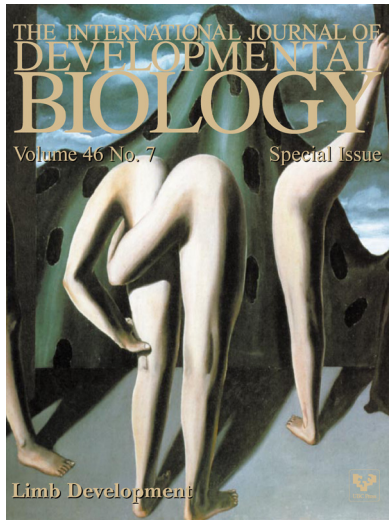

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