

Modeling Alveolar Soft Part Sarcomagenesis in the Mouse: A Role for Lactate in the Tumor Microenvironment

Matthew L. Goodwin,^{1,6} Huifeng Jin,^{1,2,6} Krystal Straessler,⁵ Kyllie Smith-Fry,^{1,2} Ju-Fen Zhu,^{1,2} Michael J. Monument,^{1,2} Allie Grossmann,³ R. Lor Randall,^{1,2} Mario R. Capecchi,^{4,5} and Kevin B. Jones^{1,2,*}

¹Department of Orthopaedics

²Center for Children's Cancer Research at the Huntsman Cancer Institute

³Department of Pathology

⁴Howard Hughes Medical Institute

⁵Department of Human Genetics

University of Utah, Salt Lake City, UT 84112, USA

⁶Co-first author

*Correspondence: kevin.jones@hci.utah.edu

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SUMMARY

Alveolar soft part sarcoma (ASPS), a deadly soft tissue malignancy with a predilection for adolescents and young adults, associates consistently with t(X;17) translocations that generate the fusion gene *ASPSCR1-TFE3*. We proved the oncogenic capacity of this fusion gene by driving sarcomagenesis in mice from conditional *ASPSCR1-TFE3* expression. The completely penetrant tumors were indistinguishable from human ASPS by histology and gene expression. They formed preferentially in the anatomic environment highest in lactate, the cranial vault, expressed high levels of lactate importers, harbored abundant mitochondria, metabolized lactate as a metabolic substrate, and responded to the administration of exogenous lactate with tumor cell proliferation and angiogenesis. These data demonstrate lactate's role as a driver of alveolar soft part sarcomagenesis.

INTRODUCTION

Approximately one-third of all sarcomas, the cancers of connective tissue, associate with chromosomal translocations that generate fusion genes (Helman and Meltzer, 2003). Many of these fusion genes have been shown to serve as primary drivers of sarcomagenesis (Haldar et al., 2007; Keller et al., 2004; Pérez-Losada et al., 2000; Straessler et al., 2013). Alveolar soft part sarcoma (ASPS), a deadly soft tissue malignancy of enigmatic origin, consistently demonstrates a t(X;17)(p11.2;q25) translocation that produces the fusion gene *ASPSCR1-TFE3* (Ladanyi et al., 2001).

ASPS presents most frequently in the adolescent or young adult limb, often metastasizes, and generally proves resistant

to chemotherapy (Folpe and Deyrup, 2006). First described in 1951 (Smetana and Scott, 1951), these highly vascular tumors demonstrate characteristic histomorphology, comprised of organoid nests of polygonal tumor cells separated by fibrovascular septa with delicate capillary arcades (Folpe and Deyrup, 2006). ASPSs also demonstrate distinct, granular structures in the cytoplasm of some cells (Ladanyi et al., 2002).

Using the few available cell lines and analysis of human ASPS tumor specimens, it has been shown that the *ASPSCR1-TFE3* fusion protein functions as a master regulatory transcription factor, joining a yet-to-be deciphered activation motif from *ASPSCR1* to the DNA-binding domain of the *TFE3* transcription factor from the MITF family (Covell et al., 2012; Kummar et al.,

Significance

By conditionally expressing in mice the fusion gene *ASPSCR1-TFE3* from human alveolar soft part sarcoma (ASPS), we generated a model that recapitulates the human tumor histologically and by expression profile, enabling study of the conditions supportive of tumor development. Mouse tumors demonstrated angiogenic gene expression in the frank absence of hypoxia and were restricted to the tissue compartments highest in lactate. They expressed high levels of lactate transport proteins (MCT1s) and responded to exogenous lactate with robust proliferation and angiogenesis. Such lactate metabolism in a malignancy has been previously postulated, but never before available for direct investigation in an in vivo tumorigenesis model. Interventions targeting lactate metabolism may provide therapeutic approaches for this and other malignancies.

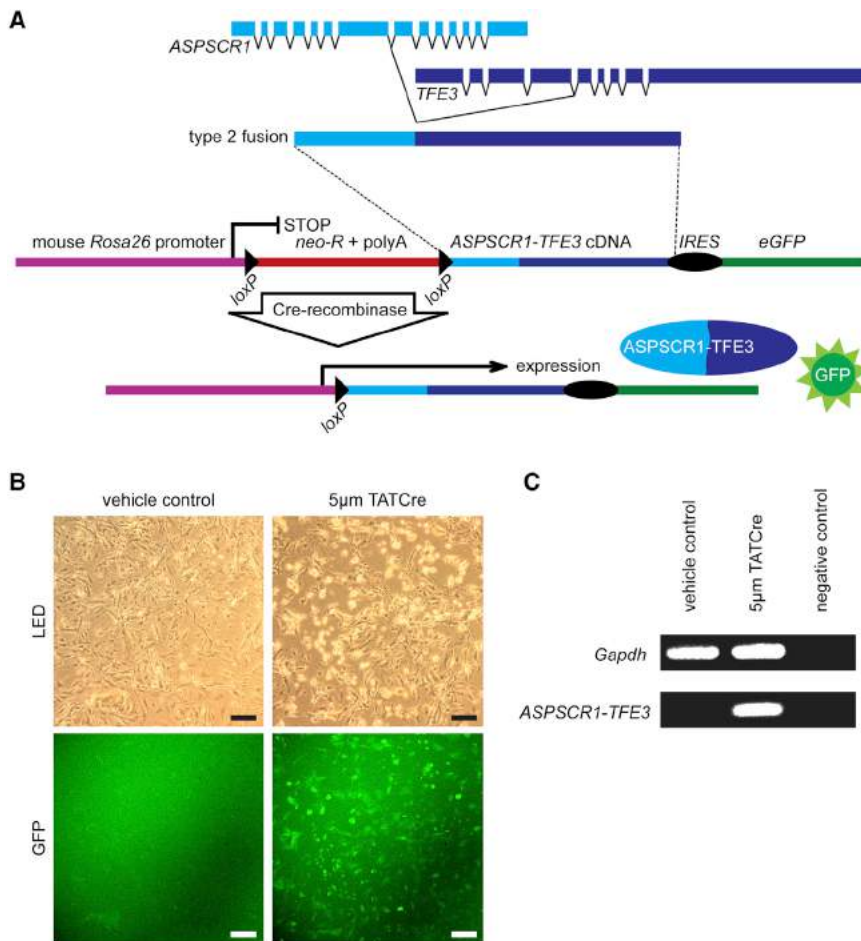


Figure 1. A Mouse Allele with Conditional Expression of Human *ASPSCR1-TFE3*

(A) Schematic demonstrating generation of the fusion transcript in human alveolar soft part sarcoma, which was reverse transcribed to a complementary DNA (cDNA) and targeted to the *Rosa26* locus, separated from its promoter by a loxP-flanked stop sequence consisting of the neomycin resistance gene (*neoR*) and a polyadenylation transcription termination sequence. The cDNA is followed by enhanced green fluorescent protein (eGFP) and an internal ribosomal entry site (IRES), such that both the fusion gene and eGFP will be translated from the same transcript following Cre-mediated recombination to remove the stop.

(B) Photomicrographs of embryonic day 12.5 *Rosa26^{LSL-AT3/WT}* fibroblasts 48 hr after exposure to TATCre or control, demonstrating GFP fluorescence from the recombined allele.

(C) RT-PCR showing presence of the fusion gene transcript following TATCre, but not control. Scale bars represent 100 μ m.

2013; Ladanyi et al., 2001; Stockwin et al., 2009). Although many angiogenic genes are highly upregulated in the tumors (Lazar et al., 2007; Vistica et al., 2009), few were found to be direct transcriptional targets of the fusion protein by chromatin immunoprecipitation analyses (Kobos et al., 2013). This suggests that some other factor, possibly related to metabolism or micro-environment, enhances angiogenesis in ASPS. We exploited an in vivo oncogenesis model to investigate.

RESULTS

Generation of an Inducible Mouse Allele of *ASPSCR1-TFE3*

In order to characterize the fusion gene associated with ASPS and its role in sarcomagenesis, we created a mouse allele designed to express *ASPSCR1-TFE3* conditionally. Briefly, the *Rosa26-LSL-AT3* allele was generated by isolating the type 2 *ASPSCR1-TFE3* fusion gene transcript from total RNA obtained from a human ASPS. The complete complementary coding sequence (cDNA) was placed in the *Rosa26* locus separated from the native promoter by a stop sequence, flanked by loxP sites (*LSL*), that is conditionally removed via Cre-mediated recombination. The fusion gene cDNA was followed by coding sequence for the *enhanced green fluorescent protein* (eGFP), linked by an internal ribosomal entry site (*IRES*) to

through the cell membrane and localization to the nucleus (Joshi et al., 2002; Straessler et al., 2013). After 48 hr, cells exposed to TATCre, but not control conditions, fluoresced (Figure 1B). RT-PCR also confirmed the presence of the fusion gene transcript following Cre-mediated recombination (Figure 1C).

ASPSCR1-TFE3 Expression Leads to Rapid, Anatomically Restricted Tumorigenesis

Considering possible tumorigenesis in a comparative context, mice homozygous for conditional *AT3*, as well as other mice homozygous for previously described conditional *SS18-SSX2* (synovial sarcoma) (Haldar et al., 2007) and *EWSR1-ATF1* (clear cell sarcoma) (Straessler et al., 2013) were bred to mice bearing a tamoxifen-inducible Cre-recombinase also driven from the *Rosa26* locus (Mao et al., 1999). It has been previously noted that in the absence of tamoxifen, a small portion of cells in most tissues in the mouse will undergo Cre-mediated recombination from nuclear entry of the CreER (Haldar et al., 2007; Straessler et al., 2013). We used it in this fashion as a low-prevalence, but random tissue initiator of expression.

In the comparative cohorts, *Rosa26^{LSL-EWSR1-ATF1}/CreER* mice demonstrated clear cell sarcoma mimicking tumors across a broad anatomic distribution in the 3–6 month age range. *Rosa26^{LSL-SS18-SSX2}/CreER* mice manifested a similar anatomic distribution of synovial sarcoma mimicking tumors, but following

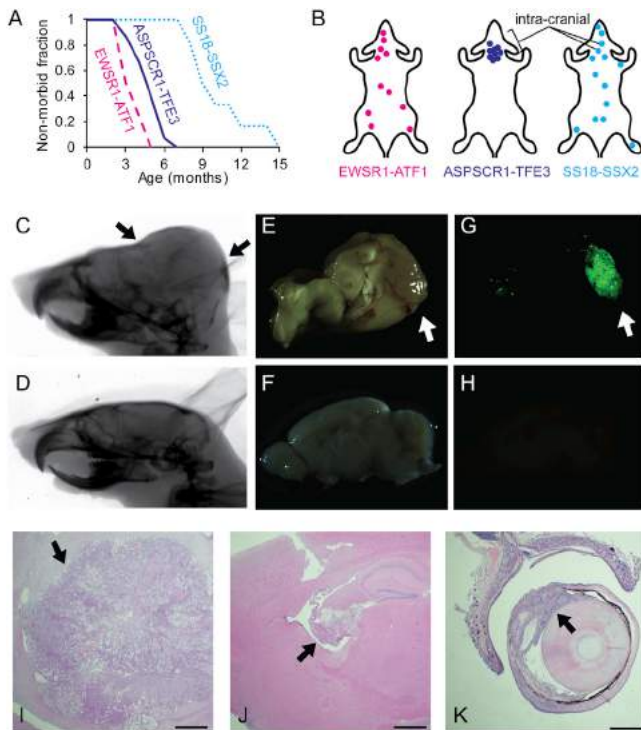


Figure 2. *ASPSCR1-TFE3* Induces Rapid Tumorigenesis Restricted to the Cranium

(A) Chart depicting the relative rates of tumorigenesis among *Rosa26^{-LSL-EWSR1-ATF1}/CreER*, *Rosa26^{-LSL-SS18-SSX2}/CreER*, and *Rosa26^{-LSL-ATF3}/CreER* mice.

(B) Diagrams of the anatomic distribution of tumors among *Rosa26^{-LSL-EWSR1-ATF1}/CreER*, *Rosa26^{-LSL-SS18-SSX2}/CreER*, and *Rosa26^{-LSL-ATF3}/CreER* mice.

(C–K) Lateral cranium radiographs (C and D), gross dissection midsagittal brain photos (E and F), and GFP fluorescence photos (G and H) of 3-month-old *Rosa26^{-LSL-ATF3}/CreER* (C, E, and G) and *Rosa26^{-LSL-ATF3}/WT* (D, F, and H) mice showing occipital expansion (between black arrows, C) from a tumor that developed in the brain (white arrows, E and G) and demonstrates GFP fluorescence from the active *ATF3* allele. Representative photomicrographs of the gamut of intracranial tumor presentations in *Rosa26^{-LSL-ATF3}/CreER* mice, in which most were intraparenchymal (I), but one encroached on the choroid plexus (J) and another developed within the eye (K). Scale bars represent 500 μm.

a longer latency of closer to a year. *Rosa26^{-LSL-ATF3}/CreER* mice developed either unusual behavior or a visibly enlarging occiput between 3 and 6 months of age. On necropsy, every mouse was noted to have developed a tumor within the skull (Figures 2A and 2B).

Rosa26^{-LSL-ATF3}/CreER mice often demonstrated enlargement of the occipital bone from inner soft tissue expansion (Figures 2C and 2D). Slightly pink colored tumors (Figures 2E and 2F) were found primarily in the brain parenchyma, but also in the choroid plexus and orbit (Figures 2I–2K). The *ATF3*-induced tumors fluoresced (Figures 2G and 2H), indicating eGFP and therefore fusion gene expression.

Mouse Tumors from *ASPSCR1-TFE3* Mimic Human ASPS Histopathology

We next determined whether or not the tumors induced by the conditional *ATF3* allele recapitulated human ASPS histopatholog-

ically. Hematoxylin and eosin (H&E) staining of a panel of human ASPS and *Rosa26^{-LSL-ATF3}/CreER* mouse tumors demonstrated the characteristic nests of polygonal cells with an open chromatin pattern, surrounded by neatly arcading capillary and larger vascular channels (Figures 3A–3D). The species of origin for each slide was microscopically indistinguishable by an expert sarcoma-focused pathologist.

Diastase treatment of sections followed by periodic acid Schiff (dPAS) histochemical staining produces a pathognomic pattern in ASPS. In mouse tumors, dPAS demonstrated the characteristic granular deposits classically described in human ASPS (Figures 3E and 3F).

Many clinical laboratories use immunohistochemical staining against TFE3 as a surrogate marker for the fusion (Argani et al., 2003). We submitted human ASPS, *Rosa26^{-LSL-ATF3}/CreER*, and *Rosa26^{-LSL-SS18-SSX2}/CreER* tumor sections to our clinical laboratory. Both human and mouse tumors associated with *ASPSCR1-TFE3* demonstrated the diagnostic, strong nuclear TFE3 staining (Figures 3G and 3H), whereas mouse tumors driven by *SS18-SSX2* provided the appropriate negative control (data not shown).

Tumors induced by conditional *ATF3* expression in the mouse often arose in or near the leptomeninges, along sulci (Figure 3I). Some tumors showed a broad, pushing front that maintained the pia mater layer between tumor and surrounding brain parenchyma (Figure 3J). Others demonstrated more aggressive invasive growth, with distinct extensions of tumor cells into the cerebellum, cerebrum, and the vessels of the choroid plexus (Figures 3K–3M). Invasive foci were often invested with capillaries (Figure 3N), which may represent tumor-induced vessels or microvascular invasion.

The *ASPSCR1-TFE3*-Driven Mouse Tumor Transcriptome Mimics Human ASPS

In order to further validate the tumors generated by conditional *ATF3* expression as a model for human ASPS, we performed a series of transcriptomic analyses. Iterative alignment of gene expression profiles between mouse and human tumors from multiple series has proven fruitful in the past to identify the unique expression signature of a given tumor type (Haldar et al., 2007). Such analyses are best performed with a common control tissue as a sounding board or background for each tumor transcriptome. Because most human ASPSs arise within skeletal muscles, and high quality muscle samples could be obtained from both mice and humans, we selected skeletal muscle as our comparative control, even though it was clear that the mouse tumors did not arise from muscle.

Sequencing of cDNA libraries prepared from total RNA (RNAseq) from five histologically and translocation-confirmed human ASPSs, three human skeletal muscle samples, five *Rosa26^{-LSL-ATF3}/CreER*-induced mouse tumors, and three mouse skeletal muscle samples yielded species-specific alignments that could be compared after filtering and reassigning mouse genes onto human homologs. Cross-species gene set enrichment analysis (GSEA) was then performed for the 500 most significantly upregulated and 500 most significantly downregulated genes in each intraspecies tumor to muscle comparison. In both directions, GSEAs showed strong and significant enrichment for the upregulated gene sets (false discovery rate [FDR]

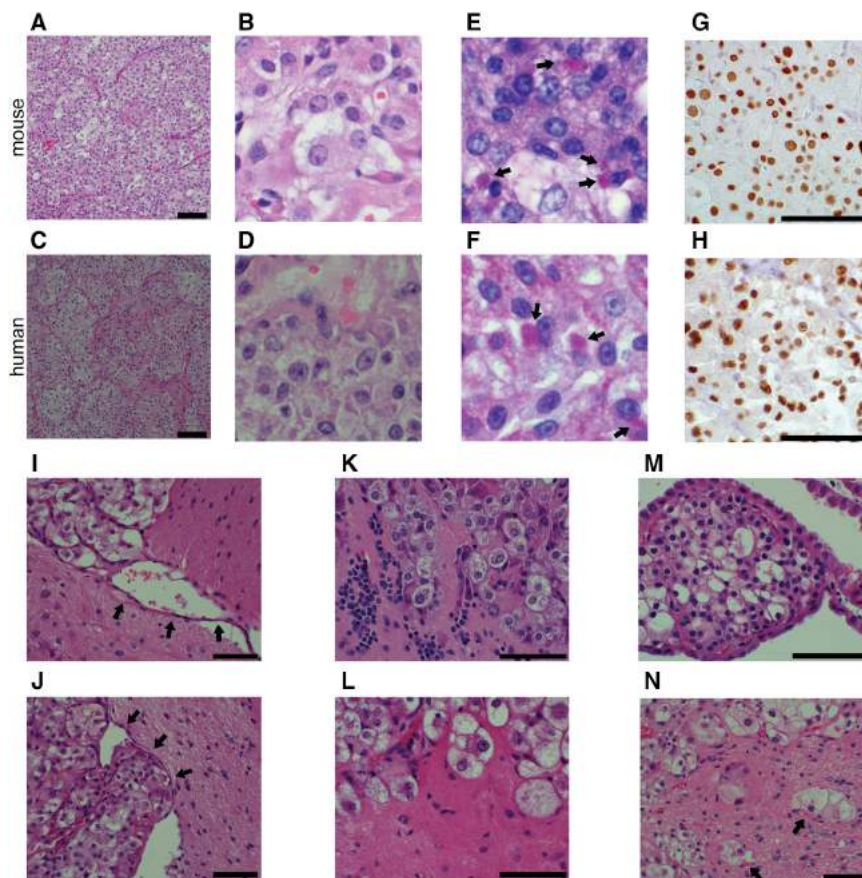


Figure 3. ASPSCR1-TFE3-Induced Mouse Tumors Recapitulate Human Alveolar Soft Part Sarcoma Diagnostic Histopathology

(A–D) Low-power (A) and high-power (B) hematoxylin and eosin photomicrographs from tumors arising in *Rosa26^{-LSL-AT3}/CreER* mice match those from human ASPSs (C and D) in the overall tissue architecture of loosely nesting cells between arcing capillary networks and cellular features of round large nuclei with prominent nucleoli.

(E and F) Photomicrograph of diastase-resistant periodic acid Schiff staining identifies the pathognomonic cytoplasmic granules (black arrows) in *Rosa26^{-LSL-AT3}/CreER* mouse tumors (E) and human ASPSs (F).

(G and H) Clinical immunohistochemistry against human TFE3 demonstrates strong nuclear staining in both *Rosa26^{-LSL-AT3}/CreER* mouse tumors (G) and human ASPSs (H).

(I–N) H&E photomicrographs demonstrate a tumor growing between sulci near the investing pia mater (arrows in I), which was sometimes maintained between the tumor cells and the surrounding tissues (arrows in J); other invading tumor cells into the cerebellum (K), cerebral cortex (L), and choroid plexus (M) maintained no intervening leptomeningeal layer, but often associated closely with small vessels (arrows in N). All scale bars and the width of (B) and (D)–(F) represent 40 μ m.

q value < 0.0005 for each GSEA) and no enrichment for the downregulated gene sets (FDR q value = 1.0 for all) (Figure 4A). This was repeated for the second through fifth groups of 500 most upregulated genes from the human RNAseq comparison in the mouse tumor to muscle gene ranking in the mouse samples, such that all genes at least 2-fold significantly upregulated were tested. All five of these gene sets were significantly enriched in the mouse tumor to muscle gene ranking with a normalized enrichment score (NES) of >1.8 and an FDR q value of <0.0005 for each. Such enrichment of homologous genes suggests a strong transcriptomic match between the mouse model and human ASPS.

For an external validation, microarray-derived expression profiles on the Affymetrix Human Genome U133 Plus 2.0 Array from seven human ASPSs and five human skeletal muscle control samples were obtained from the Gene Expression Omnibus (GEO) records GSE13433 and GSE34111. Each list of upregulated genes was significantly enriched by GSEA in comparison across species and RNAseq versus microarray platforms (FDR q value < 0.0005 for each). Each list of downregulated genes showed no enrichment (FDR q value = 1.0 for each) (Figure 4A). As a second external validation, we evaluated the expression of the 51 human genes found by Kobos et al. (2013) to be upregulated in multiple human series and whose promoters were found to be bound by the fusion oncoprotein in chromatin immunoprecipitation experiments. Forty-five of these genes were at least 1.5-fold upregulated in mouse tumors compared to mouse muscles.

In order to determine a species transcendent *ASPSCR1-TFE3* gene expression signature, we identified all human genes or mouse homologs that were at least 2-fold upregulated and statistically significant ($p < 0.05$) in all three tumor to muscle comparisons (Table S1 available online). A heatmap of these 539 unique genes, contrasting log₂ transformed fold-changes of tumors versus controls (Figure 4B) showed consistently high expression across individual tumor samples from each group. Finally, assessment of the character of these consistently upregulated genes by GO-Elite analysis noted carbohydrate metabolism, cell division, and cell cycle as the most significant biological process gene ontologies (Figure 4C).

ASPSCR1-TFE3 Expression Leads to Spatially Restricted Tumorigenesis Even among Different Cell Lineages

The observation that tumors in *Rosa26^{-LSL-AT3}/CreER* mice recapitulated human ASPS features so clearly by histopathology and transcriptomic analysis only enhanced our notice of the difference between the two species in anatomic distribution. Whereas most human ASPSs arise in skeletal muscle, none of the *Rosa26^{-LSL-AT3}/CreER* mice developed tumors in the skeletal muscle. This contrasted with our comparison mouse genetic cancer models that actually favored development of tumors within skeletal muscles, despite expressing their respective fusion genes from the identical locus and initiated by the same driver of Cre-recombinase (Figure 2B).

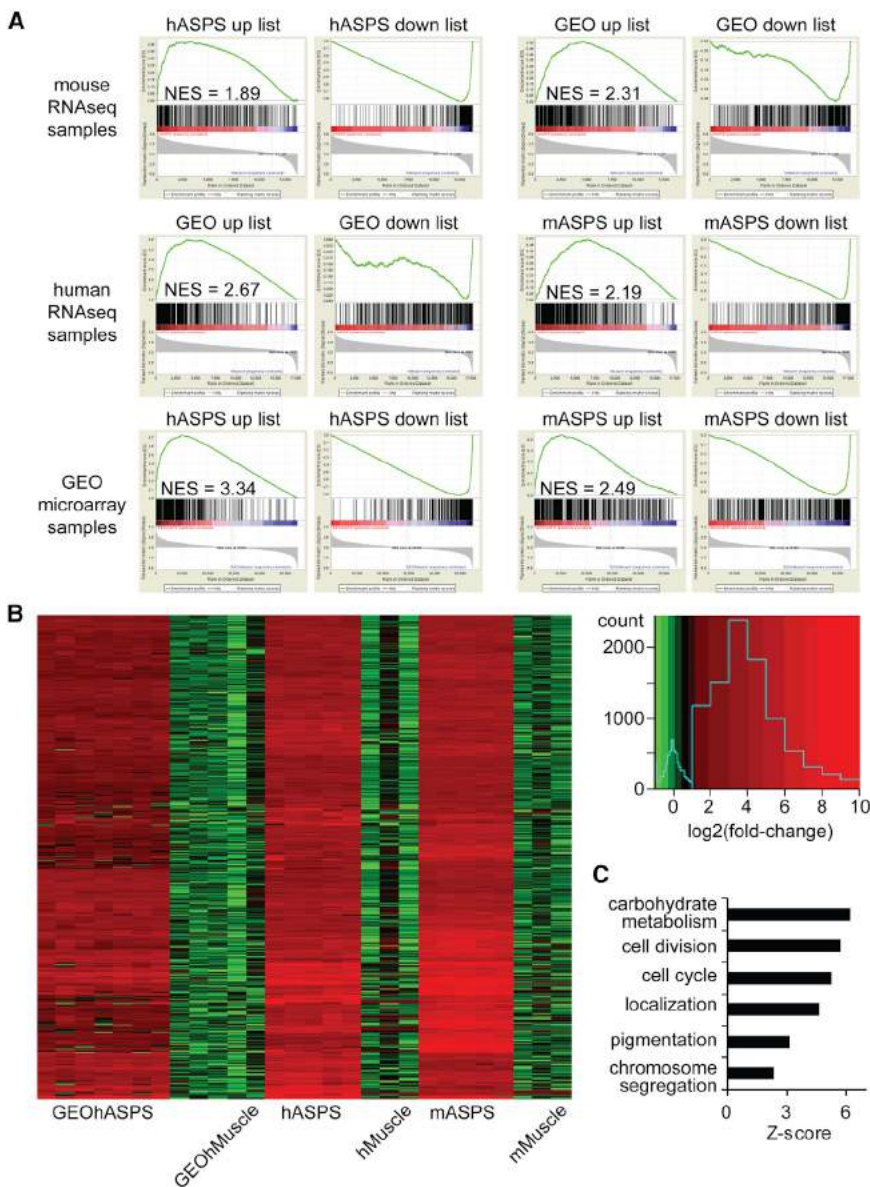


Figure 4. ASPSCR1-TFE3-Induced Mouse Tumors Recapitulate Human Alveolar Soft Part Sarcoma by Transcriptome Profile

(A) Series of Broad Institute gene set enrichment analysis (GSEA) plots testing the 500 most significantly upregulated (left, in each pair) and downregulated (right, in each pair) genes from the *Rosa26^{-LSL-AT3}/CreER* mouse tumor (mASPS) to muscle RNAseq comparison, the human alveolar soft part sarcoma (hASPS) to muscle RNAseq comparison, and the human ASPS to muscle microarray comparison (GEO), in each of the other data sets. All of the upregulated gene lists had high normalized enrichment scores (NES) and were statistically significant with false discover rate *q* values < 0.0005. None of the downregulated gene lists showed enrichment.

(B) Heat map of log₂ transformed fold-changes from the control mean of the 539 genes with at least 2-fold and significant mean upregulation in all three data sets.

(C) Chart of the Z scores of the most significant biological process gene ontologies from GO-Elite analysis of the 539 genes upregulated in all three data sets.

See also Table S1.

Suspecting that the mouse brain might harbor some preferred cell of origin for transformation by *AT3* expression, leading to tumorigenesis that was too rapid to allow slower limb tumors to become detectable, we next crossed mice bearing conditional *AT3* with mice bearing a lineage-restricted tamoxifen-inducible Cre-recombinase, expressed from a *Prx1* promoter (*Prx1-CreERT2*) (Hasson et al., 2007). *Prx1-CreERT2* mice have a well-documented lineage that emphasizes osteochondral progenitors in the periosteal mesenchyme, but also includes some intramuscular pericytes (Kawanami et al., 2009; Logan et al., 2002; Straessler et al., 2013). *Prx1-CreERT2*;*Rosa26^{-LSL-AT3}/WT* mice consistently developed intracranial tumors 10–14 weeks after tamoxifen at age 2 weeks. Interestingly, tumors were located either within the brain parenchyma or in the periosteum along the inner table of the cranium (Figure 5A). No tumors were identified in the outer table periosteum or the limbs, where most of the postnatal *Prx1* lineage is found.

ferentiation) should not be different morphologically on the inner surface of the cranium than outside the cranium.

To compare the presence of the lineage on the inner table of the cranial periosteum, we crossed *Prx1-CreERT2* bearing mice to mice bearing a *Rosa26^{-LSL-LacZ}* reporter allele (Soriano, 1999). X-gal staining of 3-week-old *Prx1CreERT2*;*Rosa26^{-LSL-LacZ}/WT* mouse brains post receipt of tamoxifen at 2 weeks demonstrated periosteal staining along the inner and outer tables of the cranium as well as in the limb periosteum (Figure 5B).

Notably, the extracranial periosteum stained just as consistently as did the intracranial periosteum with lineage tracing, but only the latter gave rise to tumors in *Prx1CreERT2*;*Rosa26^{-LSL-AT3}/WT* mice. The spatially restricted development of periosteal tumors intra- but not extracranially suggested that something unique to the microenvironment inside the cranial vault enhanced at least the efficiency of tumorigenesis there.

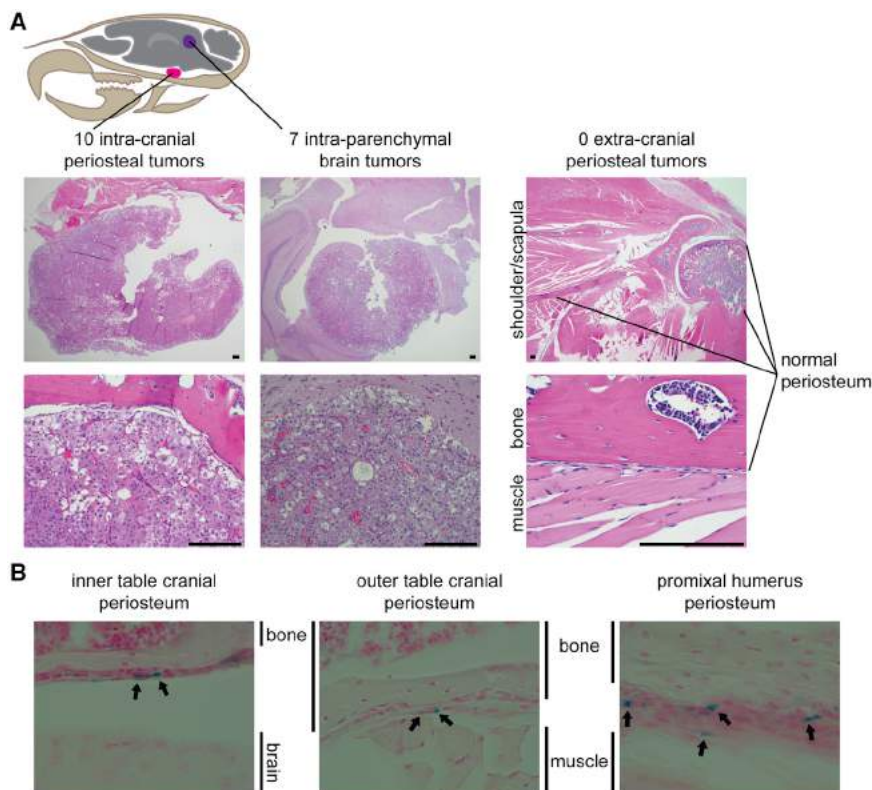


Figure 5. *ASPSCR1-TFE3*-Induced Tumorigenesis Is Most Efficient within the Cranium

(A) Diagram depicting the relative incidence and representative histopathology of the two grouped anatomic locations of tumors arising between ages 13 and 16 weeks among 6 *Prx1-CreERT2*; *Rosa26^{-LSL-AT3}/WT* littermate mice after receiving tamoxifen at age 2 weeks.

(B) Photomicrographs of X-gal-stained sections from the cranium's inner and outer tables, as well as the proximal humerus in 3-week-old *Prx1-CreERT2*; *Rosa26^{-LacZ}/WT* mice after receiving tamoxifen at age 2 weeks, demonstrating the *Prx1-CreERT2* lineage in each location (blue cells identified by black arrows), only the first of which led to identifiable tumorigenesis when initiating the *AT3* allele. Scale bars in (A) and panel widths in (B) represent 100 μ m.

compared to any other monocarboxylate transport genes in the tumors (Figure 6A). This fit the previously reported immunohistochemical finding of these two proteins in the cytoplasmic crystals stained by dPAS in human ASPS (Ladanyi et al., 2002). Further, *Mct4*, the transport gene associated with lactate exportation, was not only expressed at a much lower level

in tumors than the import-associated complex, but also at a significantly lower FPKM than in muscle.

MCTs are not pumps; their function requires lactate available for import. We therefore biochemically measured the lactate concentration in a panel of tissues in normal 2-month-old mice. Brain and eye, the host tissue compartments of all the *AT3*-driven tumors, demonstrated the highest tissue lactate concentrations (Figure 6B).

Lactate Serves as a Metabolic Substrate in *ASPSCR1-TFE3* Tumors

In order for imported lactate to contribute to metabolism, the cell would have to have significant oxidative capacity. As a measure of the abundance of mitochondria, we examined the expression of genes coding for proteins confirmed to locate within mitochondria, using the Broad Institute's MitoCarta list. Again, the comparison control of muscle was fortuitous in that muscle exhibits one of the highest mitochondrial contents in normal mammalian tissues. Surprisingly, *ASPSCR1-TFE3*-driven tumors demonstrated upregulation of nearly the entire complement of mitochondrial genes compared to quadriceps muscle controls, in both mouse and human comparisons (Figures 7A and 7B).

We also measured the oxygen consumption of fresh tissue sections from *AT3*-driven mouse tumors compared to other tumor controls, both at baseline in minimal media and following administration of lactate substrate. *ASPSCR1-TFE3*-driven mouse tumors were more oxidative than comparison mouse tumor types at baseline and responded oxidatively to the administration of lactate substrate (Figures 7C and 7D).

ASPSCR1-TFE3 Tumors Are Primed to Import Lactate

To triangulate our thinking about the microenvironment pertinent to intracranial mouse tumors and human ASPSs in skeletal muscle, we returned to what both species' tumors had in common: deranged carbohydrate metabolism (Figure 4C). Both brain and muscle have been the primary tissues of focus in the elucidation over recent decades of the phenomenon termed lactate flux (Brooks and Gaesser, 1980; Gladden, 2008). Some cells either temporarily or consistently export lactate produced by glycolysis. Other cells (in nearby or distant tissues accessed through the circulation) import and metabolize that lactate. While muscle can demonstrate a high volume lactate flux during activity, the brain consistently moves lactate between cells via the astrocyte-neuron shuttle (Aubert et al., 2005).

Significant lactate import requires elevated interstitial or intravascular lactate and the expression of monocarboxylate transporters (MCTs). Although lactate can cross membranes via other means, MCTs are responsible for the bulk of lactate transport. MCT1 and its binding partner CD147 are highly expressed in tissues that primarily import lactate; MCT4 is highly expressed in tissues that primarily export lactate (Halestrap, 2013).

We assessed the raw RNAseq expression levels (in fragments per kilobase of exon per million fragments mapped or FPKM units) of monocarboxylate transport genes in tumors and muscles. The muscle provided an ideal comparison control tissue, as skeletal muscle is known to express high levels of lactate transport genes. The two genes comprising the lactate transport complex associated with lactate importation, *Mct1* and *Cd147*, were highly overexpressed in tumors compared to muscles or

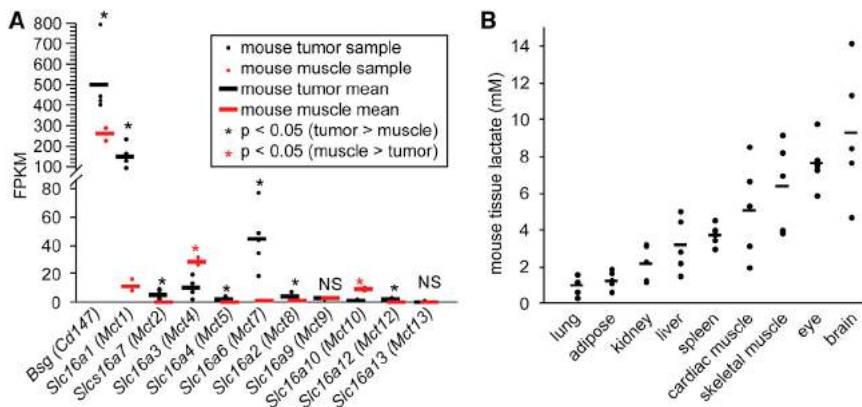


Figure 6. ASPSCR1-TFE3-Induced Mouse Tumor Lactate Transport Gene Expression and Tissue Lactate Levels in the Mouse

(A) Chart of the gene expression by fragments per kilobase of exon per million fragments mapped (FPKM) in *Rosa26^{-LSL-AT3}/CreER* mouse tumors (black) and muscle samples (red), showing high and significantly upregulated expression of both *Cd147* and *Mct1* in tumors and low and significantly downregulated expression of *Mct4*.

(B) Plot of the individual (dots) and mean (bars) concentrations of lactate in the tissues of five mice, determined biochemically.

Lactate Serves as a Signaling Molecule in ASPSCR1-TFE3 Tumors

While considered to be induced by hypoxia through downregulation of its hydroxylation and proteasomal degradation at low oxygen levels, HIF1 α also directly responds to lactate levels within a cell (Figure 8A) (Burns and Wilson, 2003; Kumar et al., 2007; Xiong et al., 1998). Needless to say, this phenomenon contributes to the hypoxic stabilization of HIF1 α , as intracellular lactate accumulates in hypoxia, but does not strictly require hypoxia itself.

It has been previously shown that human ASPS has high levels of HIF1 α (Kenney et al., 2011; Stockwin et al., 2009; Vistica et al., 2009). If these levels were generated in response to imported lactate, as opposed to lactate produced within the cell, they should be independent of hypoxia. We quantitated the level of hypoxia within the tumors by administering pimonidazole to tumor-bearing *Rosa26^{-LSL-AT3}/CreER* mice prior to euthanasia. Only in the presence of hypoxia (oxygen tensions lower than 10 torr) will pimonidazole form adducts with thiol groups that are detectable by immunohistochemistry with an available monoclonal antibody (Rosenberger et al., 2009).

Control tissues of the kidney, liver, brain, and skin were collected as well as tumors from each mouse. While the normal prevalence of pimonidazole adducts and therefore hypoxia was detected in each of the control tissues, few tumors demonstrated any hypoxia (Figure 8B). Tumors demonstrated less hypoxia than even the surrounding normal brain parenchyma.

Normal tissues showed demonstrable Hif1 α immunohistochemistry only in hypoxic regions, such as the renal tubules. In contrast, tumors demonstrated strong nuclear Hif1 α staining throughout (Figure 8B), despite normoxia. Signaling from imported lactate can increase levels of Hif1 α in normoxia. We further tested these thoroughly nonhypoxic tumors for expression of a “hypoxic” gene signature and found them to demonstrate enrichment (Figure 8C), possibly reflecting other indirectly lactate-responsive genes.

In order to test the hypothesis that *ASPSCR1-TFE3* tumors receive signaling from imported lactate, we administered lactate or saline by daily isotonic intraperitoneal (i.p.) injections for 2 weeks prior to sacrifice in a subset of mice that had developed tumors. Exogenous lactate drove increases in proliferative index and vascular density (Figure 8D-F). As cross-validation of this effect, we cultured the only two described human cancer cell lines

that express *ASPSCR1-TFE3*, FU-UR-1 (Ishiguro et al., 2004; Kobos et al., 2013) and ASPS-1 (Kenney et al., 2011) in increasing concentrations of sodium lactate, identifying a proliferative response. We further confirmed that increased exogenous lactate concentration in normoxia drove higher HIF1 α stabilization, evidenced by its increased nuclear presence. This effect was blocked by tandem application of α -cyano-4-hydroxycinnamate (CHC), a chemical inhibitor of MCT1.

DISCUSSION

We report a genetic model of alveolar soft part sarcomagenesis and have validated its mimicry of human tumors with both histologic and transcriptomic analyses. This model revealed an unusual anatomic distribution, which has implications with regard to cell of origin and the metabolic microenvironment. Despite the already reported high expression of Hif1 α and proangiogenic genes in ASPS, we demonstrated that these tumors lack frank hypoxia. When provided exogenous administration of lactate, the tumors responded with enhanced proliferation and angiogenesis, suggesting that lactate, not hypoxia, contributes to this unique form of sarcomagenesis.

Previously, members of our group have demonstrated the oncogenicity of the fusion genes that derive from chromosomal translocations in alveolar rhabdomyosarcoma (*PAX3-FKHR*) (Keller et al., 2004), synovial sarcoma (*SS18-SSX2*) (Haldar et al., 2007), and clear cell sarcoma (*EWSR1-ATF1*) (Straessler et al., 2013). Others have shown a similar transformation in the mouse by the *FUS-CHOP* fusion oncogene associated with myxoid liposarcoma (Pérez-Losada et al., 2000). By using a Cre/loxP conditional construct in the *Rosa26* locus to drive expression of *ASPSCR1-TFE3*, we have now shown this fusion gene to be sufficient for completely penetrant oncogenesis in the mouse.

The *AT3*-driven mouse tumors were histopathologically well-matched to human ASPS, demonstrating the prototypical, classic features. Expression profiles were also strikingly parallel between mouse and human tumors. Such interspecies comparisons have been fraught with difficulty in the past. We have previously utilized RNAseq as a less platform-constrained means of understanding the transcriptome of mouse tumor models (Straessler et al., 2013), but never before had human tumor RNAseq data for comparison, as we had in these analyses. In

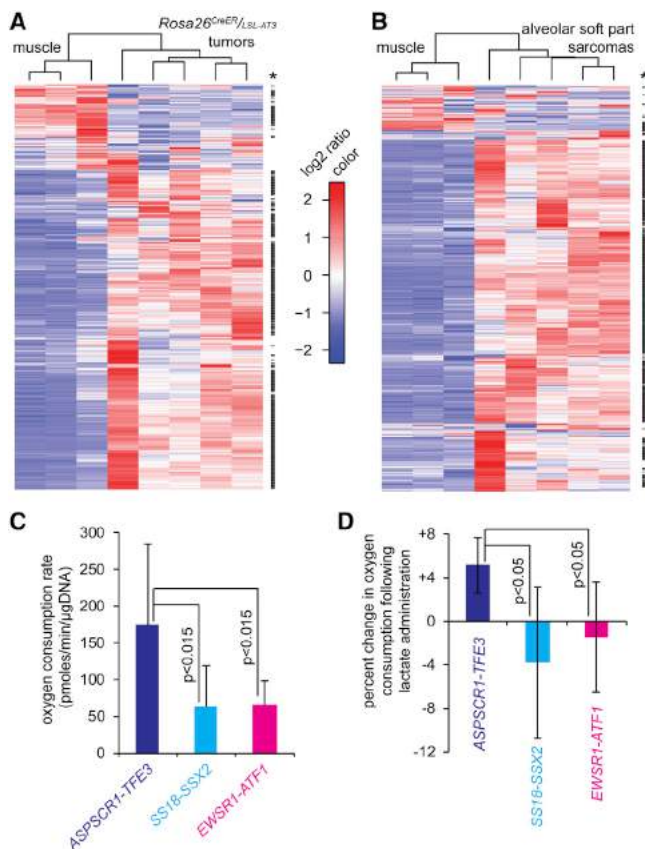


Figure 7. ASPSCR1-TFE3-Induced Mouse Tumors and Human Alveolar Soft Part Sarcomas Can Metabolize Lactate

(A and B) Heat maps from *Rosa26^{CreERT2}/LSL-ATF3* mouse tumors (A) and human alveolar soft part sarcomas (B) showing the log₂ ratios of expression normalized to the mean for the Broad Institute's curated MitoCarta gene list. (C) Chart of the mean ± SD of the oxygen consumption rate (normalized by DNA content) of tissue sections from tumors arising in mice induced by each of three fusion oncogenes, representing alveolar soft part sarcoma, synovial sarcoma, and clear cell sarcoma (n = 10 for each).

(D) Chart of the mean ± SD of the percent change in oxygen consumption among fresh tissue sections from three mouse tumor types upon administration of 10 mM lactate substrate (n = 5 for each).

addition to our primary data, we also incorporated a prior microarray-based study of human ASPS gene expression and another chromatin immunoprecipitation analysis to further validate our primary RNAseq results.

Taken together, these results from both gene expression and histopathology argue that in a manner similar to synovial sarcoma and clear cell sarcoma, the complicated biology of ASPS initiation is recapitulated by mere expression of its fusion gene *ASPSCR1-TFE3*. Importantly, none of these genetic mouse models driven by expression of translocation-generated fusion oncogenes fully recapitulates the most advanced clinical stages of its related human sarcoma. In addition to the lack of overt metastases, shared by all of the above models, this ASPS model also lacks histologically clear vascular invasion, which is commonly identified in the human counterpart, but complicated by the intracranial location of the mouse tumors. Possibly, such aggressive features of disease require more time or larger tumor

size than can be endured by mice, but notably, this emphasizes that the biology modeled is that of sarcoma initiation rather than advanced clinical disease.

While the cell of origin of this unique tumor has long been controversial, our studies lend some insights to the debate. Prior arguments have typically centered on either a myogenic or neural cell of origin, depending on gene expression in tumors as evidence of histogenesis. The formation of mouse tumors in intracranial tissue compartments that completely lack skeletal muscle removes the possibility of an exclusively skeletal muscle cell of origin. Of course, the same can be deduced from the human ASPSs described to have arisen primarily within the cranial vault (Bodi et al., 2009; Das et al., 2012) or cardiac muscle (Luo et al., 2008). Instead of confirming either muscle or neural origins, our experiments with the *Prx1* lineage argue that some mesenchymal progenitor, possibly pericyte/endothelial in character, provides one potential cell of origin distinct from either previously considered lineage. Because pericytes are ubiquitous throughout the body, the specific locations of tumors could not be explained by the availability of a cell of origin alone. However, a pericyte cell of origin in humans could explain the occasional ASPS tumor reported to arise in an unusual anatomic location.

We interpret the anatomic location of mouse tumors induced by expression of *ASPSCR1-TFE3* as circumstantial evidence of a preference for high environmental lactate. While this predilection for origin within the brain in the mouse is not recapitulated in human ASPSs, all the tissues that play common host to human ASPS are also thought to be high lactate tissues, such as skeletal muscle, cardiac muscle, and brain. Most human ASPSs arise in the skeletal muscle of active adolescents and young adults, a host tissue with high lactate shuttling. Among all sarcomas, ASPS is the most frequently (per its low prevalence) either metastatic or primary to the brain (Fox et al., 2009). While the prevalence of ASPS comprises ~1% of all sarcomas, it is the most common among primary or metastatic cardiac sarcomas in clinical series as well (Agaimy et al., 2012; Kumar et al., 2011). Interestingly, evidence from different experimental approaches has shown that the heart can get up to 60% of its energy from lactate oxidation (Gertz et al., 1988; Gladden, 2008; Stanley, 1991). While ASPS is not strictly considered to arise in the kidney—another high lactate tissue—a special subset of renal cell carcinomas express and are thought to be driven by the *ASPSCR1-TFE3* fusion gene and other TFE3 fusions (Argani et al., 2001). Much of ASPS biology has actually been inferred from studies in a clear cell carcinoma cell line that expresses *ASPSCR1-TFE3* (Ishiguro et al., 2004).

Some other information can be gleaned regarding lactate in the microenvironment from prior attempts to directly xenograft human ASPSs into the subcutaneous flanks of immunocompromised mice. Attempts with 12 different tumors yielded four successes at a latency of 8–9 months; three of those successes derived from lung or lymph node metastases (Vistica et al., 2009). While these data argue that ASPS can grow in host tissues lacking overtly high lactate, growth may be less efficient in those settings and may only be possible in select subclones.

Others have shown that lactate can contribute to angiogenesis (Semenza, 2008; Sonveaux et al., 2008). In glycolytic glioma tumor cells in vitro, lactate exposure increased HIF1 α levels independent of hypoxia by inhibiting HIF1 α proline hydroxylation,

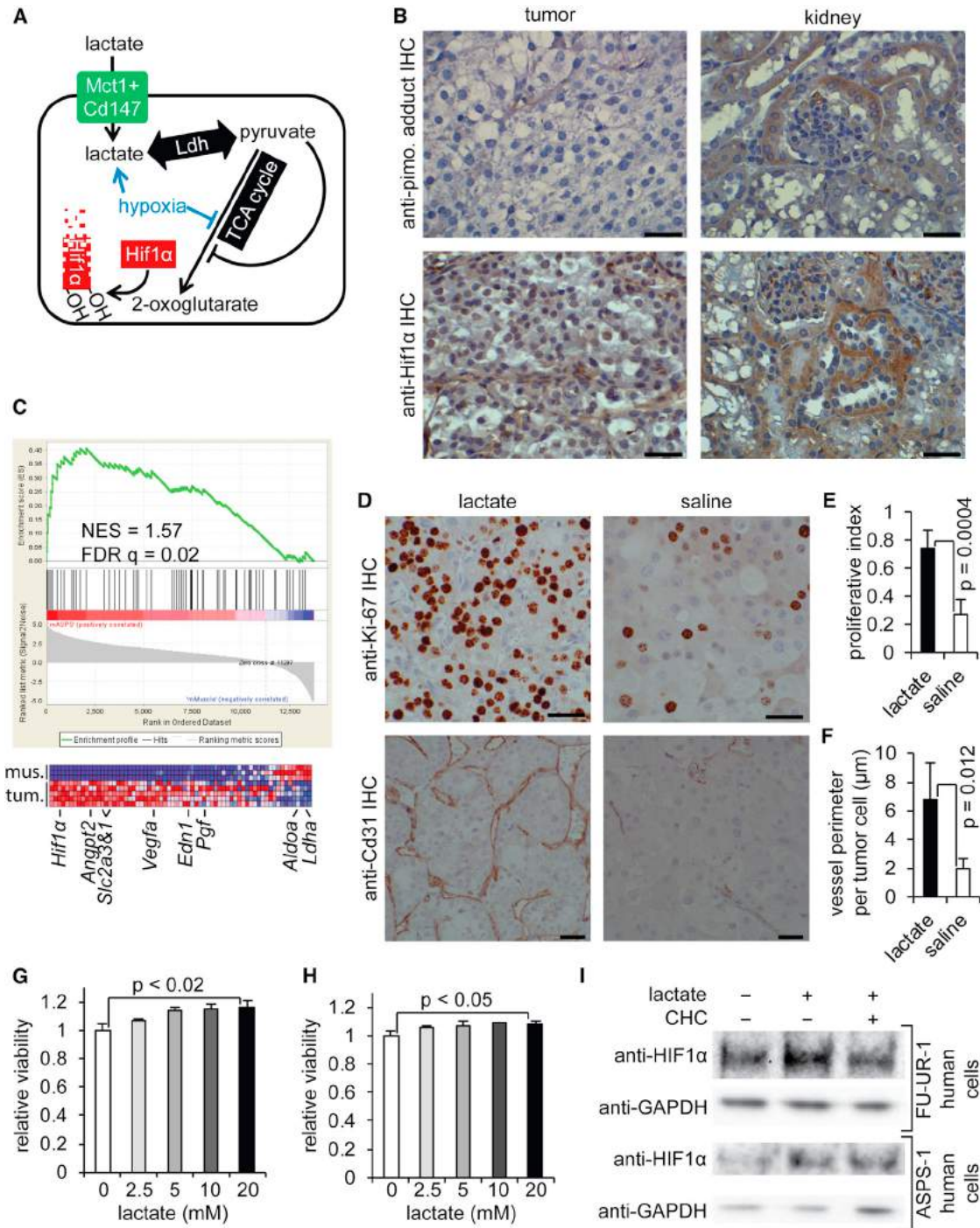


Figure 8. ASPSCR1-TFE3-Induced Mouse Tumors Express Hypoxia Genes in the Absence of Hypoxia and Respond to Exogenous Lactate (A) Explanatory diagram of the pathway by which either hypoxia or imported lactate can decrease 2-oxoglutarate-mediated Hif1α hydroxylation and degradation (Lu et al., 2002, 2005).

(B) Photomicrographs of pimonidazole-treated *Rosa26-LSL-AT3/CreER* mouse tumor and kidney tissue sections stained with immunohistochemistry against pimonidazole adducts (above; brown depicts presence of hypoxia) and Hif1α (below), demonstrating nuclear Hif1α, but no hypoxia in tumors, and Hif1α correlating to hypoxia in normal renal tubules. Scale bars represent 10 μm.

(C) Broad Institute gene set enrichment analysis for the established Harris hypoxia gene list in the *Rosa26-LSL-AT3/CreER* mouse tumor (mASPS) to muscle RNAseq comparison, showing upregulation of many ostensibly hypoxia-driven genes in the tumors.

(D) Representative photomicrographs of immunohistochemistry against Ki67, indicating proliferating nuclei (brown) or Cd31, indicating vascular endothelial cells (brown) after 2 weeks of lactate or saline administration to *Rosa26-LSL-AT3/CreER* mice bearing tumors. Scale bars represent 10 μm.

(E) Chart of the mean ± SD of the ratio of Ki-67-positive tumor cell nuclei to total tumor cell nuclei between the two groups (n = 5 lactate, n = 7 saline controls).

(legend continued on next page)

leading to increased VEGF production (Lowry et al., 1983; Lu et al., 2002, 2005). Others have shown that oxidative tumor cells in vitro activate HIF1 α via importation of lactate (De Saedeleer et al., 2012). i.p. lactate administration in mice has enhanced xenografted tumor metastasis and vascularity (Bonuccelli et al., 2010).

ASPS has long been known to be highly vascular and to express many angiogenic factors, such as HIF1 α (Lazar et al., 2007; Vistica et al., 2009). We have now added to that observation the confirmation that ASPS is also normoxic. ASPSs express high levels of lactate transporters that associate with lactate import (*Mct1* and *Cd147*) but not export (*Mct4*) and have abundant mitochondria. Not only do tumors form in areas of high lactate flux both in our model and in humans, but providing exogenous lactate also increased angiogenesis and tumor cell proliferation. Human cell lines known to express *ASPSCR1-TFE3* proliferated in response to exogenous lactate and increased in nuclear HIF1 α .

Several cancer metabolism investigations have emphasized the poor prognostic impact of hypoxia and high lactate, but these also typically associate with transitions to more metabolically quiescent cellular states (Brizel et al., 2001; Walenta et al., 1997, 2004). This is not so with ASPS, which oxidatively metabolizes lactate and increases proliferation in response to exogenous lactate.

For years, scientists have understood HIF1 α to be induced by hypoxia (Semenza, 2012). In a similar light, lactate was also long considered to be elevated only in hypoxia. Both paradigms are shifting. There is an abundance of evidence that suggests that lactate is the primary intermediate in whole body metabolism, as is best described by the lactate shuttle (Brooks, 2002). Further, in vitro studies within the last year have shown lactate to be a powerful driver of HIF1 α induction in normoxic conditions (De Saedeleer et al., 2012). We were able to test directly the degree to which increasing lactate in normoxia is an angiogenic signaling molecule driving tumorigenesis in vivo.

To conclude, ASPS is a deadly and rare tumor that portends a poor prognosis. In our efforts to characterize this particular tumor and open the door for metabolic treatments, we have shown that the tumor microenvironment can impact tumor formation and angiogenesis, specifically in regard to the role of lactate.

EXPERIMENTAL PROCEDURES

Mice

All mouse work was performed with the approval of the University of Utah institutional animal care and use committee and in accordance with international legal and ethical norms. The *EWSR1-ATF1*, *SS18-SSX2*, and *LacZ* mice were previously described (Haldar et al., 2007; Soriano, 1999; Straessler et al., 2013), as were *Rosa26-CreER* and *Prx1-CreERT2* mice (Badea et al., 2003; Hasson et al., 2007). The full length cDNA of type 2 *ASPSCR1-TFE3* was reverse transcribed from total RNA isolated from a human ASPS. The *Rosa26-LSL-AT3* targeting vector was electroporated into R1 mouse embryonic stem cells, which were selected, screened, and microinjected

into blastocysts. Please see the Supplemental Experimental Procedures for additional details.

Clinical

With the approval of the University of Utah institutional review board and in accordance with all international legal and ethical standards, fresh surgical specimens from consenting patients were banked and stored, then analyzed after annotation with histopathological, molecular, and clinical data. Please see the Supplemental Experimental Procedures for additional details.

Transcriptome Analysis

Sequencing was performed on an Illumina HiSeq 2000 (Illumina) using a 50 cycle single end read after mRNA capture by the Ribo-Zero method. The *OverdispersedRegionScanSeqs* script in the *USeq* package (Nix et al., 2008) calculated the expression levels of ENSEMBL genes in FPKM values. *USeq* also discovers differentially expressed genes between two RNA-seq libraries by calling the *DESeq* R package (Anders and Huber, 2010).

Gene Expression Omnibus (GEO) data were normalized by DChip (Li and Hung Wong, 2001). GSEA was performed with Broad Institute software (Mootha et al., 2003; Subramanian et al., 2005). The Harris hypoxia gene set is curated by the Broad Institute (Harris, 2002), as is MitoCarta, including the original 1,098 mouse genes, whose proteins localize to the mitochondria, and their 1,013 human homologs (Pagliarini et al., 2008). Gene ontology analysis was performed with GO-Elite software (Zamboni et al., 2012). Please see the Supplemental Experimental Procedures for additional details.

ACCESSION NUMBERS

The Gene Expression Omnibus (GEO) accession number for the sequencing data reported in this paper is GSE54729.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ccell.2014.10.003>.

AUTHOR CONTRIBUTIONS

M.L.G. and H.F. focused on lactate related experiments and the mouse allele targeting, respectively. The project was conceived by K.B.J.. Experiments were performed by M.L.G., H.F., K.S., K.S.F., J.F.Z., and M.J.M. A.G. provided pathological expertise. The manuscript was written by M.L.G. and K.B.J. and edited by all authors.

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(F) Chart of the mean \pm SD of the ratio of the linear vessel perimeter to total tumor cell nuclei between the two groups (n = 5 lactate, n = 7 saline controls). (G and H) Charts presenting mean \pm standard deviation of the relative viability of ASPSCR1-TFE3-expressing human cancer cell lines FU-UR-1 (at 48 hr, G) and ASPS-1 (at 72 hr, H) in increasing concentrations of sodium lactate (n = 3 for each point; experiments independently repeated three times). (I) Western blots showing increased nuclear HIF1 α when exposed to 20 mM sodium lactate that is blocked by the MCT1-inhibitor, CHC at 0.1 mM in FU-UR-1 cells and 1 mM in ASPS-1 cells.

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