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Limb bud and flank mesoderm have distinct "physical phenotypes" that may contribute to limb budding

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ABSTRACT

Limb bud outgrowth in chicken embryos is initiated during the third day of development by Fibroblast Growth Factor 8 (FGF8) produced by the newly formed apical ectodermal ridge (AER). One of the earliest effects of this induction is a change in the properties of the limb field mesoderm leading to bulging of the limb buds from the body wall. Heintzelman et al. [Heintzelman, K.F., Phillips, H.M., Davis, G.S., 1978. Liquid-tissue behavior and differential cohesiveness during chick limb budding. J. Embryol. Exp. Morphol. 47, 1–15.] suggested that budding of the limbs is caused by a higher liquid-like cohesivity of limb bud tissue compared with flank. We sought additional evidence relevant to this hypothesis by performing direct measurements of the effective surface tension, a measure of relative tissue cohesivity, of 4-day embryonic chicken wing and leg bud mesenchymal tissue, and adjacent flank mesoderm. As predicted, the two types of limb tissues were 1.5- to 2-fold more cohesive than the flank tissue. These differences paralleled cell number and volume density differences: 4-day limb buds had 2- to 2.5-fold as many cells per unit area of tissue as surrounding flank, a difference also seen at 3 days, when limb budding begins. Exposure of flank tissue to exogenous FGF8 for 24 h increased its cell number and raised its cohesivity to limb-like values. Four-day flank tissue exhibited a novel and unique active rebound response to compression, which was suppressed by the drug latrunculin and therefore dependent on an intact actin cytoskeleton. Correspondingly, flank at this stage expressed high levels of α-smooth muscle actin (SMA) mRNA and protein and a dense network of microfilaments. Treatment of flank with FGF8 eliminated the rebound response. We term material properties of tissues, such as cohesivity and mechanical excitability, the "physical phenotype", and propose that changes thereof are driving forces of morphogenesis. Our results indicate that two independent aspects of the physical phenotype of flank mesoderm can be converted to a limb-like state in response to treatment with FGF8. The higher tissue cohesivity induced by this effect will cause the incipient limb bud to phase separate from the surrounding flank, while the active mechanical response of the flank could help ensure that the limb bud bulges out from, rather than becoming engulfed by, this less cohesive tissue.

Introduction

The morphogenesis of the vertebrate limb begins with the bulging of the limb buds from the body wall at four sites along the embryo's surface. In the avian embryo, the lateral plate mesoderm is induced by paraxial signals (Saito et al., 2006) to generate limb buds at the axial levels of the cervical-thoracic (forelimb or wing) and lumbosacral (hindlimb or leg) somites by the third day of development (Nowicki et al., 2003). The mesenchymal cells of the limb buds and adjacent flank are spatially homogeneous and morphologically similar until sub-populations within the limb buds begin to condense into cartilaginous primordia and premuscle masses more than a day later.

Exposure of the limb field mesoderm to Fibroblast Growth Factor-8 (FGF8) and/or other FGFs secreted from the apical ectodermal ridge (AER) is essential in promoting limb bud outgrowth (Mahmood et al., 1995; Vogel et al., 1996; Crossley et al., 1996; Ohuchi et al., 1997; Lewandoski et al., 2000; Moon and Capecchi, 2000; Sun et al., 2002). The limb vs. flank difference, however, is manifested in avian and mammalian species even earlier than the initial bulging (Min et al., 1998; Xu et al., 1998; Sekine et al., 1999; Kawakami et al., 2001; Agarwal et al., 2003; Tanaka and Tickle, 2004; Saito et al., 2006).

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How might changes in the limb field cells lead to bulging of the tissue from the surrounding flank? Much evidence suggests that on the time scale of typical morphogenetic processes many embryonic tissues behave similarly to viscous liquids. As a consequence, they exhibit characteristic liquid mechanical properties, including rounding up into droplet-like spheres, cohesivity and surface tension, and coalescence and miscibility/immiscibility (Steinberg and Poole, 1982; Armstrong, 1989; reviewed in Forgacs and Newman, 2005). Based on these ideas and indirect evidence from cell sorting and tissue fragment coalescence experiments, Phillips and his coworkers (Heintzelman et al., 1978) made the novel suggestion that limb budding occurs because limb field mesoderm becomes more cohesive than the somatopleural mesoderm from which it arises. According to this hypothesis, a limb bud would emerge from the body wall by a physical process akin to that which causes a droplet of water to separate and protrude from the surface of a pool of oil.

Recently, compression tensiometry for measuring the biomechanical properties of tissues directly has become available (Foty et al., 1996; Forgacs et al., 1998; Hegedűs et al., 2006; Norotte et al., 2008). In this method, small fragments of the tissue are allowed to round up in suspension culture, compressed between parallel plates and let to return to mechanical equilibrium. During the compression and subsequent relaxation process, forces exerted by the tissue on the plates, as well as the tissue's exact shape, are monitored. The analysis of the force–time curve by well-established physical models provides reproducible values for the tissue's surface tension and viscoelastic parameters (Forgacs et al., 1998; reviewed in Forgacs and Newman, 2005; Norotte et al., 2008).

We have utilized these methods to further test the plausibility of the hypothesis of limb budding of Heintzelman et al. (1978). Here we report that at 4 days of development both wing and leg bud mesenchyme had higher surface tensions (i.e., were more cohesive) than flank tissue. The magnitude of the difference between limb and flank was similar to that measured in other pairs of embryonic tissues which behaved like immiscible liquids (Foty et al., 1996). A cellular correlate of the observed cohesivity difference between flank and limb mesoderm is the difference in cell density, which we show by morphometric analysis to be more than twice the flank value in limb buds, both at 4 days, when the compression experiments were performed, and at 3 days, when limb budding is initiated. Significantly, in relation to the hypothesis of Heintzelman et al. (1978), we found that exogenous application of FGF8 induced the flank mesoderm to increase its cell number, and to bring its surface tension to a value within the range of the limb bud mesoderms.

In the course of these studies we discovered that the flank mesoderm (which contains no myoblasts at these stages), exhibited an unusual active mechanical response during the first two minutes of compression, manifested by a sudden increase of the force exerted by the tissue on the compression plate. This rebound response has not been reported in any of the more than a dozen embryonic tissue types and aggregates of genetically modified tissue culture cells analyzed in the same fashion in earlier studies (Foty et al., 1996; Forgacs et al., 1998; Duguay et al., 2003). We found it to be dependent on an intact actin cytoskeleton in flank tissue, and correlated with high levels of expression of α-smooth muscle actin (SMA) (compared to trace amounts in the limb buds) and an extensive network of cytoplasmic microfilaments. Treatment with FGF8 elevated the cohesivity of the flank to limb-like values and completely eliminated the rebound response. However, the low cohesivity of flank tissue relative to limb bud was not due to the cytoskeletal features responsible for the rebound response, since elimination of the latter by treatment with the drug latrunculin had no effect on the tissue's cohesivity.

We conclude that cohesivity (high in limb bud, low in flank) and the ability to mount an active mechanical response (absent in limb bud, present in flank) are two independent features of the "physical phenotype" of these tissues (see also Newman and Comper, 1990). Changes toward limb-like values in each of these features are induced by FGF8 and both plausibly contribute to the induction of limb buds from the flank tissue during embryogenesis.

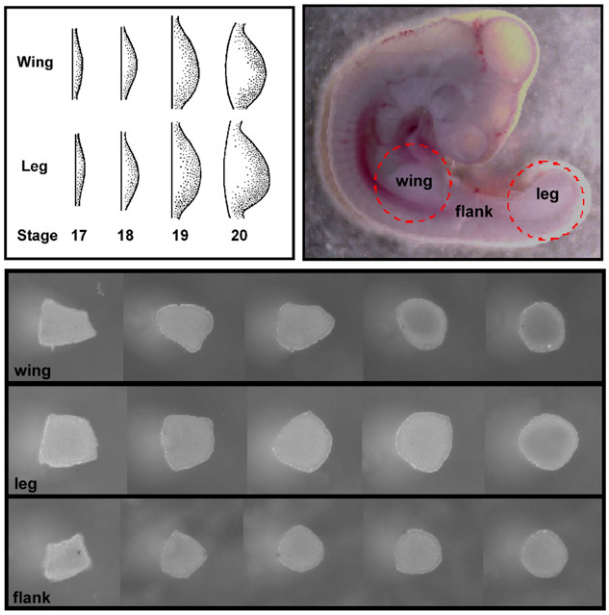

Fig. 1. (Top left) Changes in the shape of chicken wing and leg buds as the limb buds emerge from the flank between HH17 (~58 h) and HH20 (~71 h). (Redrawn from Hamburger and Hamilton, 1951, with modifications.) (Top right) E4 (HH23) chicken embryo, showing sources of tissue used in this study. (Bottom) The rounding of fragments (~300 μm) of E4 leg, wing and flank tissue occurs within 24 h in vitro, an indication of their liquid-like behavior. The fragment in the left-most panel of each row was photographed shortly after explantation. The fragments were photographed successively 5 h, 11.5 h, 17.5 h and 24 h later.

Materials and methods

Embryos, tissues and reagents

Fertilized White Leghorn eggs (Ozark Hatcheries, Neosho, MO for tensiometry experiments, Moyers Chicks, Quakertown, PA for histologic, ultrastructural, and DNA and RNA analyses) were incubated at 38.5 °C with 80% humidity until Hamburger–Hamilton stage HH23 (Hamburger and Hamilton, 1951) was reached (4 days, Fig. 1, upper right panel). Dissections of wing and leg buds, and flank (somatopleural) tissue between them, were performed in cold (21–23 °C) Earle's Balanced Salt Solution without calcium or magnesium (EBSS, Invitrogen). A solution of EBSS with 2 mM ethylene diamine tetra-acetic acid (EDTA, Invitrogen) was used to chelate calcium, allowing layers of ectodermal cells to be gently removed. The action of the EDTA was halted using a solution of EBSS with 1.8 mM $CaCl_2$ and 0.8 mM $MgSO_4$ + 10% Fetal Bovine Serum (EBSS + FBS, Invitrogen). Tissue explants were cut into ~300 μm fragments and incubated where indicated for ~24 h in Dulbecco's Modified Eagles Medium containing 1% Penicillin Streptomycin (DMEM, Invitrogen). FBS (10%) was added to the medium for all tensiometric and some other assays, as indicated. Recombinant mouse fibroblast growth factor 8b (FGF8b, R&D Systems) was used at a concentration of 25 ng/ml and latrunculin A (Invitrogen) at 1 μM. Trypan blue, used in the post-compression viability assay, was purchased from Invitrogen.

Tensiometry

Incubation in the presence of FBS caused the tissue fragments to round into spheres (Fig. 1, bottom). Tissue surface tension was measured with an in-house built compression plate tensiometer (Fig. 2, left). The tensiometer's inner chamber is maintained at 37 °C and contains CO_2 independent medium with 10% FBS and 1% penicillin